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(54) **Title:** COMPOSITIONS AND METHODS OF SYNTHESIS AND USE OF NOVEL NUCLEIC ACID STRUCTURES

(57) **Abstract:** The present invention is directed to compositions of nucleic acid structures that can be used in molecular biological techniques. Additionally, the present invention is directed to methods of making such nucleic acid structures and to methods of using such nucleic acid structures. These compositions and methods can be used in such molecular biological techniques including, but not limited to, diagnostic tests involving triplex and duplex formations, signal amplification systems, nucleic acid synthesis and others. Preferred compositions of the present invention comprise parallel-stranded oligomers having at least one 8-aminopurine in the purine strand and having the general formula of purine-linker-pyrimidine, in either orientation.

# COMPOSITIONS AND METHODS OF SYNTHESIS AND USE OF NOVEL NUCLEIC ACID STRUCTURES

## Related Applications

The present application claims the priority of U.S. Provisional Patent Application No. 60/162,627, filed October 29, 1999, and U.S. Provisional Application No.60/197,559, filed April 17, 2000.

## Technical Field

The invention relates to novel oligomer analogs and to oligonucleotide-based diagnostics by binding of the oligomers to single and double-stranded nucleic acids target sequences. More specifically, the invention concerns oligomers containing 8-aminopurine base residues and their triple-helix stabilization properties.

## Background of the Invention

It has been shown that oligonucleotides could bind to homopurine-homopyrimidine sequences of double stranded DNA by forming triple helices. The formation of nucleic acid triple helices offers the possibility of designing sequence-specific DNA binding molecules, which may have important uses as diagnostic tools as well as therapeutic treatments. For example, triple helices are used for the extraction and purification of specific nucleotide sequences, control of gene expression, mapping genomic DNA, detection of mutations in homopurine DNA sequences, site-directed mutagenesis, triplex-mediated inhibition of viral DNA

integration, nonenzymatic ligation of double-helical DNA and quantitation of polymerase chain reactions (for reviews see references 1-3).

One of the problems for the development of applications based on triple helix formation is the low stability of triple helices especially at neutral pH. Another problem associated with the use of triplex forming oligonucleotides (TFO) is the presence of interruptions in the homopurine-homopyrimidine tracks. In order to overcome this problem a lot of effort has been put into the design and preparation of modified oligonucleotides in order to enhance triple helix stability. See references 39 and 40. One of the most successful modifications is to replace natural bases with some modified bases such as 5-methylcytidine, 5-bromouracil, 5-aminouracil, $N^4$-spermine-5-methylcytidine, or 5-methyl-2,6(1H,3H)-pyrimidinedione

The most studied type of triple helix formation is the so-called purine: pyrimidine: pyrimidine motif. (Figure 1). In this motif, the purine: pyrimidine strands correspond to the target double-stranded DNA sequence (known as the Watson-Crick purine and pyrimidine strands) and the Hoogsteen strand is a pyrimidine strand used for the specific recognition of the double-stranded DNA. See U.S. Patent Nos. 5,422,251 and 5,693,471. For these reasons, most of the base analogues studied for triple helix stabilization are modified pyrimidines located at the Hoogsteen strand, though there are some recent disclosures of purine analogs. For example, see U.S. Patent Nos. 5,739,308; 5,645,985; and 5,594,121.

In order to obtain a triplex, in some occasions, purine residues are concentrated on one chain and are linked to a pyrimidine chain of inverted polarity. By "inverted polarity" is meant that the oligomer contains tandem sequences which have opposite polarity, i.e. one segment or region of sequences having polarity 5'->3', followed by another with polarity 3'->5' or vice versa. This implies that these sequences are joined by linkages which can be thought as a 3'-3' or a 5'-5'

internucleotide junction. Such oligomers named "parallel-stranded DNA" have been synthesized See References 34, 42 and 43.

Recent results have shown that the introduction of an amino group at position 8 of adenine increases the stability of triple helix due to the combined effect of the gain in one Hoogsteen purine-pyrimidine H-bond, (see references 4-6) and the ability of the amino group to be integrated into the "spine of hydration" located in the minor-Major groove of the triplex structure (Figure 2). (See references 4-7). A similar behavior has been observed with 8-amino-2-deoxyguanosine and 8-amino-2'-deoxyinosine (Figure 2). (See reference 11). The preparation and the characterization of the binding properties of oligonucleotides containing 8-aminopurines has been described, but these oligonucleotides can not be directly used for the specific recognition of double-stranded DNA sequences because the modified bases are purines that are in the target sequence and not in the Hoogsteen strand used for the specific recognition of double-stranded DNA.

Synthetic oligonucleotides probes have been proven very useful in the detection of cloned DNA sequences. When a partial protein sequence is available, a mixture of oligonucleotides presenting all possible DNA sequences can be successfully used as a probe or as PCR or sequencing primers for screening of cloned DNA (or amplification of DNA. See reference 44. The mixed probe approach may have two principal drawbacks when the complexity of the mixture is very high. First, the oligonucleotide probes must, for reasons of practicality, be synthesized together on the same support. Thus, the products of the synthesis can never be adequately characterized. Second, since the exact coding sequence is not known, it is difficult to set appropriate stringent conditions for the hybridisation and subsequent washings. A universal base—one that could base pair equally well with any of the four natural bases—could resolve these two difficulties. A number of compounds have been tested

as possible universal bases, with being 2'-deoxyinosine one of the most successfully used. See references 45-50.

Other molecular biological techniques, such as selective restriction of nucleic acids and target detection, can be improved. Though current techniques are adequate, there are inherent problems in the amplification steps. The PCR system will amplify any DNA added to the mixture, regardless of whether it contains the correct target sequence. If DNA fragments are selected based only on the size of the fragment that is created by the restriction enzymes, then the target sequence may be missed entirely. Additionally, if the restriction enzymes become contaminated, there is no assurance that the correct sequences are being restricted and that the target sequence is being selected. Obtaining and maintaining purified nucleases is often problematic in laboratory settings and is even more of a problem in automated systems.

Thus, methods and compositions are needed that are capable of specifically selecting target nucleic acid sequences that do not require amplification or that can be used with amplification techniques but provide for more target specific amplification. Additionally, what is needed are compositions and methods that require less use of enzymes.

Though several techniques are currently available for modification of nucleic acid structures, what is needed are compositions and methods for binding to specific target regions of DNA or RNA sequences. Specifically what is needed are triplex structures that are stabile at neutral pHs and modified oligonucletides that can bind to specific sequences in a target nucleic acid to form triple helix structures. Modified oligonucleotides that can be used for synthesis of oligonucleotides in the 5' to 3' direction, reverse of the normal 3' to 5' synthesis direction are also needed.

What is also needed are spacer arms that can link oligonucleotides in 5' to 5' orientation or 3' to 3' orientation. Particularly needed are simple and economic methods for the synthesis of such spacer arms and such paired oligonucleotide structures.

Compositions and methods for incorporation of modified nucleic acid bases are also needed. What is particularly needed are methods and compositions comprising a base labeled with active compounds, such as intercalating agents, photoreactive agents and cleavage agents that are attached to the base through a linker arm.

**Summary of the Invention**

The present invention is directed to methods and compositions of nucleic acid structures that are used for detection of specific nucleic acid sequences. One of the embodiments of the present invention comprises compositions and methods for the preparation of oligonucleotides carrying modified nucleic acids, such as 8-aminoadenine, 8-aminoguanine and 8-aminohypoxanthine, that are connected 3' to 3' or 5' to 5' (head-to-head or tail-to-tail) to a Hoogsteen pyrimidine strand. For example, see Figure 3. These modified oligonucleotides allow the specific recognition of double-stranded, and single-stranded nucleic acids by binding to the Watson-Crick pyrimidine strand via a triple helix. Additionally, the present invention comprises a universal base oligonucleotide that forms stable base paris with the four natural bases.

Preferred methods of the present invention comprise binding or capturing a predetermined sequence on DNA or RNA by forming a triple helix that will be stable at neutral pH. The stability of the triple helix at neutral pH allows the use of these modified oligonucleotides in conjunction with enzymatic reactions in

order to enhance the discriminatory power of the modified oligonucleotides and the direct use of these oligonucleotides under physiological conditions.

The methods and compositions of the present invention comprise use of hairpin structures to form triplex structures. The present invention also provides methods and compositions for detecting a specific nucleic acid target. A preferred embodiment comprises methods involving hybridization of hairpin probes that comprise a 5' to 3' purine sequence, followed by a loop sequence, followed by a 3' to 5' pyrimidine sequence that is complementary to the purine sequence. These self-annealing probes comprise a region, the loop, that will not hybridize to any of the probe's sequence. The loop sequence comprises any sequence that will not hybridize to itself.. At least one of the hairpin probes can be complexed with a magnetic bead or a molecule that is effective for capture or detection of the structure formed with the hairpin.

The present invention also comprises compositions and methods for making spacer arms that allow the synthesis of paired parallel stranded oligonucleotides using either 3' phosphoramidite chemistry or 5' phosphoramidite chemistry. Additionally, the oligonucleotide member of the pair an comprise any number of nucleotides.

The present invention further comprises compositions and methods for synthesis of nucleosides with linker arms for attachment of active compounds. Preferably, the active compounds include, but are not limited to, intercalation agents, photoreactive compounds and agents capable of cleaving nucleic acids. More preferably, the present invention comprises compositions and methods of synthesis and use of pyrimidine nucleosides with linker arms at the $N^4$ of 2'-deoxycytidine wherein the active compound is a protected fluorescent label and a cleavage agent, such as 5-bromouracil.

**Brief Description of the Figures**

Figure 1 is a drawing of a triple helix with the purine: pyrimidine: pyrimidine motif. Base-pairing scheme of the triads A: T: T and G : C : C+.

Figure 2 is a drawing of the base-pairing of triads containing 8-aminopurines.

Figure 3 is a drawing of a double- or single-stranded nucleic acid (RNA or DNA) binding with oligonucleotide derivatives of the present invention.

Figure 4 shows a method for making oligonucleotide sequences containing 8-aminopurines using phosphoramidite chemistry.

Figure 5 is a drawing of the triplex formed using the oligonucleotide composition, B-22A.

Figure 6 is a drawing of the triplex formed using the oligonucleotide composition, B-22A, and B-22A control where no Hoogstein bonding can occur.

Figure 7 A and B are drawings of procedures to produce 3'-3' (a) or 5'-5' (b) linker attached oligonucleotides.

Figure 8 is a drawing of the synthesis steps of the glycerol-spacer arm (S).

Figure 9 A and B is a drawing of the synthesis of the 5'-5' and 3'-3' attached oligonucleotides.

Figure 10 shows the synthesis of the glycerol-spacer arm (AS) and attachment to LCAA-CPG using a linker.

Figure 11A and B show the synthesis of 5'-5' and 3'-3' attached oligonucleotides with different sequences using the AS-glycerol spacer arm.

Figure 12 shows the synthesis of the 2-nitrophenyl intermediate.

Figure 13 illustrates a protected fluorescein attached to the 1,2-bis(aminoethoxy)ethane linker arm.

Figure 14A and B diagram the synthesis of the $N^4$-spacer arm-fluorescein labelled-2'-deoxycytidine phosphoramidite.

Figure 15 shows a method for attachment of the 5-bromouracil to the 1,2-bis(aminoethoxy)ethane linker arm.

Figure 16 A and B details the synthesis of the $N^4$-spacer arm-5-bromouracil-2'deoxycytidine phosphoramidite.

Figure 17A, B and C show the synthesis of the 8-amino-2'-deoxyadenosine monomer.

Figure 18A and B diagrams the synthesis of the 8-amino-2'-deoxyguanosine reversed monomer.

Figure 19A and B illustrates the synthesis of the 8-amino-2'-deoxyinosine reversed monomer.

Figure 20A and B show the synthesis of the 5-methyl-2'-deoxycytidine reversed monomer.

Figure 21A and B show embodiments of the methods of the present invention using the triplex forming oligonucleotides and detection of the structures formed.

**Detailed Description of the Invention**

The present invention is directed to compositions of nucleic acid structures that can be used in molecular biological techniques. Additionally, the present invention is directed to methods of making such nucleic acid structures and to methods of using such nucleic acid structures. These compositions and methods can be used in such molecular biological techniques including, but not limited to, diagnostic tests involving triplex and duplex formations, signal amplification systems, nucleic acid synthesis and others.

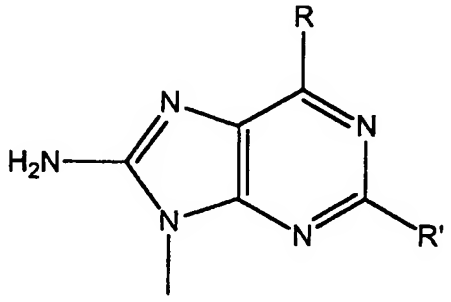
Preferred compositions of the present invention comprise parallel-stranded oligomers having at least one 8-aminopurine in the purine strand of general formula:

$^{3'}$purine strand having 8-aminopurines$^{5'}$---linker---$^{5'}$pyrimidine strand$^{3'}$

or

$^{5'}$purine strand having 8-aminopurines$^{3'}$---linker---$^{3'}$pyrimidine strand$^{5'}$

where ---linker--- comprises any method of coupling nucleotide sequences of opposite polarity. The 8-amino purines on the purine strand have the general formula indicated below.




R = OH, $NH_2$

R' = H, OH, $NH_2$

The present invention also comprises compositions and methods for the preparation of oligonucleotide derivatives constituted by a polypyrimidine part linked head-to-head with a polypurine sequence carrying several 8-aminopurines such as 8-aminoadenine, 8-aminoguanine and 8-aminohypoxanthine. Methods of use of these derivatives comprise binding specifically to a predetermined target sequence, preferably a polypyrimidine target sequence, by forming a very stable triple helix that can be observed even at neutral pH. The high degree of stabilization obtained by the addition of several 8-aminopurines is especially relevant to the development of new applications based on triple helix formation such as structural studies, DNA-based diagnostic tools, and antigene therapy.

An embodiment of the present invention comprises oligonucleotide derivatives comprising two parts: a polypyrimidine part connected head-to-head to a complementary purine part carrying one or more 8-aminopurines such as 8-aminoadenine, 8-aminoguanine and 8-aminohypoxanthine. A linker molecule is located between both parts in such a way that both parts can form a double stranded structure in parallel sense. These oligonucleotide derivatives bind polypyrimidine sequences complementary (in the antiparallel sense) to the purine part by triple helix formation. DNA triple helical structures are normally observed at acidic pH. However, when oligonucleotides carrying 8-aminopurines were used, very stable triple helical structures were observed even at neutral pH. Applications based on triple helix formation will benefit from the use of these oligonucleotide derivatives.

In another embodiment of the present invention, a polypyrimidine part is connected tail-to-tail to a complementary purine part carrying one or more 8-aminopurines.

**Structure of the olig nucleotides derivatives**

The preparation of oligonucleotide derivative sequences and binding properties of oligonucleotide derivatives carrying 8-aminoadenine, 8-aminoguanine and 8-aminohypoxanthine connected head-to-head or tail-to-tail to the Hoogsteen pyrimidine strand is taught herein. Some embodiments of the sequences of the oligonucleotides of the present invention are shown in Table 1.

Table 1

| Name | Sequence |
|---|---|
| R-22 | $^{5'}$GAAGGAGGAGA$^{3'}$-(EG)$_6$-$^{3'}$TCTCCTCCTTC$^{5'}$ |
| R-22A | $^{5'}$GAAGGA$^{N}$GGA$^{N}$GA$^{3'}$-(EG)$_6$-$^{3'}$TCTCCTCCTTC$^{5'}$ |
| R-22G | $^{5'}$GAAG**G**$^{N}$AGG$^{N}$AGA$^{3'}$-(EG)$_6$-$^{3'}$TCTCCTCCTTC$^{5'}$ |
| R-22I | $^{5'}$GAAGI$^{N}$AGI$^{N}$AGA$^{3'}$-(EG)$_6$-$^{3'}$TCTCCTCCTTC$^{5'}$ |
| B-22 | $^{3'}$AGAGGAGGAAG$^{5'}$-(EG)$_6$-$^{5'}$CTTCCTCCTCT$^{3'}$ |
| B-22A | $^{3'}$AGA$^{N}$GGA$^{N}$GGAAG$^{5'}$-(EG)$_6$-$^{5'}$CTTCCTCCTCT$^{3'}$ |
| B-22G | $^{3'}$AGA**G**$^{N}$GA**G**$^{N}$GAAG$^{5'}$-(EG)$_6$-$^{5'}$CTTCCTCCTCT$^{3'}$ |
| B-22AG | $^{3'}$AGA$^{N}$**G**$^{N}$GA$^{N}$**G**$^{N}$GAAG$^{5'}$-(EG)$_6$-$^{5'}$CTTCCTCCTCT$^{3'}$ |
| B-22Acontrol | $^{3'}$AGA$^{N}$GGA$^{N}$GGAAG$^{5'}$-(EG)$_6$-$^{5'}$**TTTTTCCCCCC**$^{3'}$ |
| B-22AMMT | $^{3'}$AGA$^{N}$GGA$^{N}$CGAAG$^{5'}$-(EG)$_6$-$^{5'}$CTTC**T**TCCTCT$^{3'}$ |
| B-22ALT1 | $^{3'}$AGA$^{N}$GGA$^{N}$GGAAG$^{5'}$-$^{5'}$**TTTT**-CTTCCTCCTCT$^{3'}$ |
| B-22ALT2 | $^{3'}$AGA$^{N}$GGA$^{N}$GGAAG-**TTTT**$^{5'}$-$^{5'}$CTTCCTCCTCT |
| B-22ALTGA | $^{3'}$AGA$^{N}$GGA$^{N}$GGAAG-**GGAGG**$^{5'}$-$^{5'}$CTTCCTCCTCT$^{3'}$ |

Oligonucleotide derivatives (R-22, R-22A, R-22G, R-22I) contain 22 bases divided in two different parts and connected with a hexaethyleneglycol linker [$(EG)_6$]. One half corresponds to a polypyrimidine sequence, that will be similar to the target polypyrimidine sequence but with the inverted orientation. This sequence will be the Hoogsteen strand in the triple helix. The other half corresponds to a polypurine sequence that will be complementary to the target polypyrimidine in which two purines are substituted by the corresponding 8-aminopurine. In the oligonucleotide R-22A, two adenines are substituted by two 8-aminoguanines ($A^N$). In the oligonucleotide R22G, two guanines are substituted by two 8-aminoguanines ($G^N$) and in the oligonucleotide R-22I, two guanines are substituted by two 8-aminohypoxanthines ($I^N$). The control oligonucleotide (R-22) contains only the natural bases without modification.

The second group of oligomers (B-22, B-22A, B-22G) are similar in composition to the previous oligomers but the polypurine and the polypyrimidine parts are connected through their 5' ends with an hexaethyleneglycol linker [(EG)6] In addition an oligomer having two 8-aminoguanines and two 8-aminoadenines was prepared (B-22AG) to test whether the stabilizing properties both 8-aminopurines are additive. Also, a control oligonucleotide (B-22Acontrol) having the same oligonucleotide sequence in the polypurine part than B-22A but a non-sense polypyrimidine sequence was prepared. Finally, the oligomer B-22AMMT was prepared to test the influence of interruptions on the stability of the triple helix. In this oligomer there are two adenines substituted by two 8-aminoadenines. In the middle of the purine part there is a pyrimidine (C) and in the corresponding position at the Hoogsteen strand there is a T.

A third group of oligomers (B-22ALT1, B-22ALT2, B-22ALGA) have the same nucleotide sequence than B-22A but the loop between the polypurine and polypyrimidines parts are made out of nucleotides (-TTTT- and –GGAGG-) instead of the hexaethyleneglycol bridge. Figure 3 is a general representation of the binding of the oligonucleotides of the present invention with single or double-stranded nucleic acids.

**Oligonucleotide synthesis**

A preferred method for synthesizing the oligonucleotides of the present invention comprises use of phosphoramidite chemistry. It is to be understood that the synthesis of the oligonucleotides herein described, or their equivalents, can be synthesized by any methods known to those skilled in the art. See References 34, 41 and 42. In a preferred method, oligonucleotide sequences containing 8-aminopurines were prepared using phosphoramidite chemistry on an automatic DNA synthesizer (see Figure 4). For the preparation of the pyrimidine part, reversed C and T phosphoramidites and reversed C-support (linked to the support through the 5' end) were used. After the assembly of the pyrimidine part, an hexaethyleneglycol linker was added using a commercially available phosphoramidite. Finally, the purine part carrying the modified 8-aminopurines was assembled using standard phosphoramidites for the natural bases and the 8-aminopurine phosphoramidites prepared as described. See references 4, 5, 10-13.

The phosphoramidite of 8-aminohypoxanthine was prepared as follows. 8-Amino-2'-deoxyadenosine was deaminated by the action of the enzyme adenosine deaminase (Adenosine aminohydrolase, EC 3.5.4.4), yielding 8-amino-2'-deoxyinosine. Protection of the 8-amino group was performed by addition of the phenoxyacetyl group. See reference 50. The resulting $N^8$-phenoxyacetyl derivative

was not soluble and was reacted with isobutyryl chloride. The resulting $N^8$-phenoxyacetyl- $N^8$-isobutyryl derivative was reacted with dimethoxytrityl chloride in pyridine yielding 8-amino-2'-deoxy-5'-O-dimethoxytrityl-$N^8$-isobutyryl-inosine. During the introduction of the dimethoxytrityl group, the phenoxyacetyl group was removed due to the lability of this group. Direct introduction of the isobutyryl group at $N^8$ position is possible but yields are lower than the route with the phenoxyacetyl group. The phosphoramidite group was introduced in position 3' using chloro-O-2-cyanoethoxy-N,N-diisopropylamino-phosphine as described. See reference 51.

After the assembly of the sequences, supports were treated with concentrated ammonia. Oligonucleotides carrying 8-aminoguanine were treated with 0.1 M 2-mercaptoethanol in concentrated ammonia See reference 13. After deprotection, the products were purified by reverse-phase HPLC using the DMT-on and DMT-off protocols. In all cases a major peak was obtained and collected. The purified oligonucleotides were analyzed by enzyme digestion followed by HPLC analysis of the resulting nucleosides. In all cases the purified oligonucleotides were obtained in good yields and had the correct nucleoside composition.

**Binding properties**

Methods for detecting formation of triplexes are contemplated by the present invention. One of the embodiments of these methods used for the detection of the formation of the triple helix structure comprise the measurement of the triplex fusion temperature, which comprises incubating the samples at increasing temperatures vs. time, and collecting hyperchromicity data. Hyperchromicity is the amount by which the UV absorbance of a nucleic acid sample increases when going, for example, from a double stranded structure to a single strand structure as it melts in response to increasing temperature.

A preferred embodiment of the assay is described below:

1. A single stranded template, 11 bases long, of constant sequence, in most cases, consisting exclusively of a pyrimidine stretch (WC-11mer).

2. The hairpin probes described above, of the same length, where the two strands are joined by a loop consisting of 6 ethylene glycol members or the sequences TTTT or GGAGG; and the purine stretch contain modified bases (i.e., 8-amino A and/or 8-amino G, or 8-amino-I).

The relative stability of triple helices formed by the R-22 oligonucleotide derivatives and the polypyrimidine target sequence (WC-11mer) were measured spectrophotometrically at different pHs (pH 5.5-7.0). In the examples shown herein, one single transition was observed with an hyperchromicity around 25% that was assigned to the melting of the triple helix. Replacement of A and G by 8-aminoadenine ($A^N$) and 8-aminoguanine ($G^N$) in triple helix results in a high stabilization of triple helix (10-18°C in the range from pH 5.5 to pH 7.0, see Table 2). Replacement of guanine by 8-aminohypoxanthine ($I^N$) gave only a small increase of triple helix stability at acidic pH but the triplex containing $I^N$ maintained their stability at neutral pH while the unmodified triplex decreases very fast.

Table 2: Melting temperatures* (°C) for the triplex formed by R-22 derivatives and WC-11mer.

| | |
|---|---|
| WC-11mer | $^{3'}$CTTCCTCCTCT$^{5'}$ |
| R-22 | $^{5'}$GAAGGAGGAGA$^{3'}$-(EG)$_6$-$^{3'}$TCTCCTCCTTC$^{5'}$ |
| R-22A | $^{5'}$GAAGGA$^{N}$GGA$^{N}$GA$^{3'}$-(EG)$_6$-$^{3'}$TCTCCTCCTTC$^{5'}$ |
| R-22G | $^{5'}$GAAGG$^{N}$AGG$^{N}$AGA$^{3'}$-(EG)$_6$-$^{3'}$TCTCCTCCTTC$^{5'}$ |
| R-22I | $^{5'}$GAAGI$^{N}$AGI$^{N}$AGA3'-(EG)$_6$-$^{3'}$TCTCCTCCTTC$^{5'}$ |

| Hairpin | Target | pH 5.5 | pH 6.0 | pH 6.5 | pH 7.0 |
|---|---|---|---|---|---|
| R-22 | WC-11mer | 56 | 47 | 35 | 32 |
| R-22A | WC-11mer | 62 | 56 | 48 | 46 |
| R-22G | WC-11mer | 67 | 60 | 53 | 51 |
| R-22I | WC-11mer | 54 | 47 | 40 | 38 |

*1 M NaCl, 100 mM sodium phosphate/ citric acid buffer.

The relative stability of triple helices formed by the B-22 oligonucleotide derivatives and the polypyrimidine target sequence (WC-11mer) were measured. In this case the purine and pyrimidine strands are connected by their 5'ends. As before, one single transition was observed with an hyperchromicity around 25% that was assigned to the melting of the triple helix. Replacement of A by 8-aminoadenine (AN) and guanine by 8-aminoguanine (GN) in triple helix results in a high stabilization of triple helix (see Table 3) as observed previously.

Table 3: Melting temperatures* (°C) for the triplex formed by B-22 derivatives and WC-11mer.

WC-11mer $^{3'}$CTTCCTCCTCT$^{5'}$

R-22 $^{3'}$TCTCCTCCTTC$^{5'}$-(EG)$_6$-$^{5'}$GAAGGAGGAGA$^{3'}$

R-22A $^{3'}$TCTCCTCCTTC$^{5'}$-(EG)$_6$-$^{5'}$GAAGGA$^{N}$GGA$^{N}$GA$^{3'}$

R-22G $^{3'}$TCTCCTCCTTC$^{5'}$-(EG)$_6$-$^{5'}$GAAGG$^{N}$AGG$^{N}$AGA$^{3'}$

| Hairpin | Target | pH 5.5 | pH 6.0 | pH 6.5 | pH 7.0 |
|---|---|---|---|---|---|
| B-22 | WC-11mer | 54 | 45 | 33 | 20 |
| B-22A | WC-11mer | 57 | 51 | 43 | 34 |
| B-22G | WC-11mer | 69 | 59 | 50 | 40 |

*1M NaCl, 100 mM sodium phosphate / citric acid buffer

In order to check that the transition was due to triple helix formation, melting curves were performed with hairpins (R-22, R-22A, R-22G, R-22I, B-22, B-22A, B-22G) alone without the presence of the polypyrimidine target sequence (WC-11 mer). In this case, a single transition was also observed but at lower temperature and with a hyperchromicity around 10-15%, indicating that the transition observed with WC-11mer (triple helix) is different from the transition observed without WC-11mer (parallel-stranded double helix). Melting temperatures are shown in Table 3.

Table 4: Melting temperatures* (°C) for the R-22 and B-22 derivatives alone.

| Hairpin | pH 5.5 | pH 6.0 | pH 6.5 | pH 7.0 |
|---|---|---|---|---|
| R-22 | 34 | 25 | -- | -- |
| R-22A | 50 | 43 | 28 | 28 |
| R-22G | 55 | 50 | 40 | 40 |
| R-22I | 42 | 34 | 25 | 23 |
| B-22 | 35 | 25 | -- | -- |
| B-22A | 47 | 38 | -- | -- |
| B-22G | 54 | 44 | 30 | -- |

*1 M NaCl, 100 mM sodium phosphate/ citric acid buffer.

The transition observed without the presence of WC-11 mer indicates that R22 derivatives have a parallel double-stranded structure. This structure is more stable at acidic pH and in the presence of 8-aminopurines (see Table 4). Because one of the structures observed in parallel-stranded DNA is a Hoogsteen base pair and this type of base pair will be stabilize by the presence of 8-aminopurines, it is theorized, though not wishing to be bound by any particular theory, that this type of base pair is the responsible of the stability of the parallel structure observed in R22 derivatives, in particular B-22A, (Figure 5). Because this structure is very similar to the structure in the triple helix (see Figure 2) the hairpin derivatives comprised in this invention have a preformed structure that will facilitate the formation of triple helices.

The role of the Hoogsteen strand was further investigated. A hairpin probe of the same purine sequence was prepared, but with two 8-aminoadenine substitutions, and with a non-complementary pyrimidine strand. This oligonucleotide (named B-22Acontrol) can only form Watson-Crick base pair with the target sequence (WC-11mer); but no Hoogsteen bond can be formed (Figure 6). As mentioned above, hyperchromicity is the amount by which the UV absorbance of a nucleic acid sample increases when going, for example, from a double stranded structure to a single strand structure as it melts in response to increasing temperature.

A 10 – 15 % increase is considered to correspond to the (melting) transition from a duplex to a single-stranded structure, in one step. A 15 – 25 % increase is considered to correspond to the (melting) transition from a triplex to a single-stranded structure, in one step.

Table 5: Effect of the Hoogsteen strand.

| Hairpin | Target | pH 5.5, 1 M NaCl | | pH 6.0, 1 M NaCl | |
|---|---|---|---|---|---|
| | | Tm (°C) | Hyperchromicity | Tm (°C) | Hyperchromicity |
| B-22Acontrol | WC-11mer | 41.3 | + 12 % (du to ss) | 40.0 | + 11 % (du to ss) |
| B-22Acontrol | none | No transition | | No transition | |
| B-22A | WC-11mer | 57.0 | + 22 % (tri to ss) | 51.0 | + 20 % (tri to ss) |
| B-22A | none | 47.0 | + 12 % (du to ss) | 38.0 | + 11 % (du to ss) |

When the Hoogsteen strand is replaced by a non-complementary sequence, the structure of parallel duplex is not observed anymore, as indicated by the disappearance of the transition observed when the melting curve is performed without the target WC-11mer.

The transitions observed with the duplexes formed by B22-Acontrol: WC-11mer have lower Tm and lower hyperchromicity. The hyperchromicity associated with the transition of duplex formed by B-22Acontrol : WC-11mer is around 11%, which indicates a transition of duplex to single-stranded.

The transitions observed with the complex formed by B22A : WC-11mer have a 22 % hyperchromicity typical of a transition from triplex to single-stranded. The difference between the Tm of B-22Acontrol duplex and B–22A triplex is the gain obtained by the addition of a complementary Hoogsteen strand. At pH 6.0,

this difference is 11 °C (1.0 degree per base) and, at pH 5.5, is 16 °C (1.4 degree per base).

The effect of the salts at pH 6.0 were studied. The triplex formed by hairpin having two 8-aminoadenines ($A^N$) and WC-11mer were used as well as the triplex formed by the hairpin having two 8-aminoguanines ($G^N$) and WC-11mer. The effect of the concentration of NaCl, $MgCl_2$, and spermine on the stability of the triplex was studied. Previous reports on triplexes showed an increased stabilization of triplexes by the addition of magnesium and spermine. See reference 2. The effect of these salts on a triplex is shown in the following tables. In the case of NaCl, the buffer used was 0.1 M sodium phosphate-citric acid pH 6.0. In the cases of $MgCl_2$ and spermine, the buffer used was 0.1 M sodium phosphate pH 6.0.

Table 6: Effect of the NaCl.

| hairpin | target | [NaCl] | Tm (°C) |
|---|---|---|---|
| B-22A | WC-11mer | 0 | 48.9 |
| B-22A | WC-11mer | 0.1 M | 50 |
| B-22A | WC-11mer | 1 M | 51 |

0.1 M sodium phosphate-citric acid pH 6.0

Table 7: Effect of the $MgCl_2$

| hairpin | target | [$MgCl_2$] | Tm (°C) |
|---|---|---|---|
| B-22A | WC-11mer | 0 | 53.2 |
| B-22A | WC-11mer | 10 mM | 58.5 |
| B-22A | WC-11mer | 50 mM | 58.4 |
| B-22G | WC-11mer | 0 | 57.6 |
| B-22G | WC-11mer | 10 mM | 62.8 |
| B-22G | WC-11mer | 50 mM | 63.4 |

0.1 M sodium phosphate pH 6.0

Table 8: Effect of spermine

| hairpin | target | [spermine] | Tm (°C) |
|---|---|---|---|
| B-22G | WC-11mer | 0 | 57.6 |
| B-22G | WC-11mer | 1 mM | 60.3 |
| B-22G | WC-11mer | 5 mM | 59.6 |

0.1 M sodium phosphate pH 6.0

Sodium chloride had a small stabilization effect (from 48.9 °C (no NaCl) to 51 °C (1M NaCl)). Low concentrations of magnesium chloride stabilized the triplex, for example the melting temperature of triplex B-22G : WC-11mer and B-22A : WC-11mer increases 5 degrees from no $MgCl_2$ to 10 mM $MgCl_2$. Going from 10 mM to 50 mM $MgCl_2$ the increase on the melting temperature was none or less than one degree. At pH 6 adding more magnesium than 50 mM resulted in precipitation of magnesium oxide during the heating. In conclusion, the presence of

the magnesium is beneficial for the stability of triplex, with 10 mM the optimal concentration. Spermine had a small stabilizing effect on the stability of the triplexes.

Next, the stabilization properties of 8-aminoadenine and 8-aminoguanine were studied to see if the effects were additive. A hairpin with two 8-aminoadenines and two 8-aminoguanines substitutions was prepared. Melting curves were performed with the following hairpins and the target WC-11mer at pH 6.0, 0.1 M sodium phosphate, 1 M NaCl.

| | | |
|---|---|---|
| B-22 | $^{3'}$AGAGGAGGAAG$^{5'}$-$(EG)_6$-$^{5'}$CTTCCTCCTCT$^{3'}$ | Tm = 45.0 °C |
| B-22A | $^{3'}$AGA$^{N}$GGA$^{N}$GGAAG$^{5'}$-$(EG)_6$-$^{5'}$CTTCCTCCTCT$^{3'}$ | Tm = 51 °C |
| B-22G | $^{3'}$AGAG$^{N}$GAG$^{N}$GAAG$^{5'}$-$(EG)_6$-$^{5'}$CTTCCTCCTCT$^{3'}$ | Tm = 59 °C |
| B-22AG | $^{3'}$AGA$^{N}$G$^{N}$GA$^{N}$G$^{N}$GAAG$^{5'}$-$(EG)_6$-$^{5'}$CTTCCTCCTCT$^{3'}$ | Tm = 65.4 °C |

The stabilization properties of the 8-aminopurines were additive. The addition of the two 8-aminoguanines and two 8-aminoadenines increased the melting temperature by 20 °C, while the increase on the melting temperature induced by two 8-aminoadenine was 6 °C, and the increase induced by two 8-aminoguanines was 14 °C.

Next, the effect of an interruption on the polypyrimine track of the target was measured. To this end a polypyrimidine target with a purine in the middle of the sequence was prepared ($s_{11}$-MM : $^{5'}$TCTCCTGCTTC$^{3'}$). Next, the following hairpin carrying two 8-aminoadenines was designed (B-22AMMT: $^{3'}$AGA$^{N}$GGA$^{N}$CGAAG$^{5'}$-$(EG)_6$-$^{5'}$CTTCTTCCTCT$^{3'}$). This oligomer has a C opposite to the G of $s_{11}$-MM and a T on the Hoogsteen strand opposite to the C:G interruption. The choice of T on the Hoogsteen strand is based on previous reports that showed

that T is the less destabilizing base in similar situations. Melting curves were performed at pH 6.0, 0.1 M sodium phosphate, 1 M NaCl.

| # | Sequence | Tm (°C) |
|---|---|---|
| B-22AMMT | $^{3'}$AGA$^{N}$GGA$^{N}$CGAAG$^{5'}$-(EG)$_6$-$^{5'}$CTTCTTCCTCT$^{3'}$<br>$s_{11}$-MM: $^{5'}$TCT CCT GCT TC$^{3'}$ | 44.5 |
| B-22AMMT | $^{3'}$AGA$^{N}$GGA$^{N}$CGAAG$^{5'}$-(EG)$_6$-$^{5'}$CTTCTTCCTCT$^{3'}$<br>WC-11mer: $^{5'}$TCT CCT CCT TC$^{3'}$ | 30.1 |
| B-22A | $^{3'}$AGA$^{N}$GGA$^{N}$GGAAG$^{5'}$-(EG)$_6$-$^{5'}$CTTCCTCCTCT$^{3'}$<br>$s_{11}$-MM: $^{5'}$TCT CCT GCT TC$^{3'}$ | 42.8 |
| B-22A | $^{3'}$AGA$^{N}$GGA$^{N}$GGAAG$^{5'}$-(EG)$_6$-$^{5'}$CTTCCTCCTCT$^{3'}$<br>WC-11mer: $^{5'}$TCT CCT CCT TC$^{3'}$ | 51 |

The introduction of an interruption on the polypyrimidine-polypurine track was studied. A guanine was introduced on the polypyrimidine target instead of a cytosine. An hairpin with two 8-aminopurines was designed in where a cytosine was located in the purine part opposite to the cytosine. A thymine was located in the Hoogsteen pyrimidine part. The hairpin B-22AMMT was able to bind to its target ($s_{11}$-MM) although there is a decrease of 6 degrees in the Tm (44.5 °C B-22AMMT : $s_{11}$-MM compared with 51 °C B-22A : WC-11mer). The binding of the new hairpin to its new target was very selective as judged by the big decrease observed on the Tm of the triplex B-22AMMT with the old target (30.1 °C B-22AMMT : WC-11mer versus 44.5 °C B-22AMMT : $s_{11}$-MM). In conclusion, the oligomers of the present invention can be redesigned to cope with the presence of interruptions on the polypurine- polypyrimidine tracts.

Finally, the role of the loop on the stability of the triplex was analysed by preparing derivatives of B-22A with different loops. In addition to the hexaethyleneglycol linker, the nucleotide loops –TTTT- and –GGAGG- were studied. See reference 41. Two tetrathymine loops were prepared : one on reversed orientation than the purine strand (B-22ALT1) and the second on the same orientation than the purine strand (B-22ALT2). The GGAAA loop was on the same orientation than the purine strand (B-22ALGA). Melting curves were performed with the following hairpins and the target WC-11mer at pH 6.0, 0.1 M sodium phosphate, 1 M NaCl.

| # | Sequence | Tm (°C) |
|---|---|---|
| B-22A | $^{3'}$AGA$^{N}$GGA$^{N}$GGAAG$^{5'}$-(EG)$_6$-$^{5'}$CTTCCTCCTCT$^{3'}$ | 51 |
| B-22-ALT1 | $^{3'}$AGA$^{N}$GGA$^{N}$GGAAG$^{5'}$-$^{5'}$TTTT-CTTCCTCCTCT3' | 57 |
| B-22ALT2 | $^{3'}$AGA$^{N}$GGA$^{N}$GGAAG-TTTT$^{5'}$-$^{5'}$CTTCCTCCTCT$^{3'}$ | 55 |
| B-22ALTGA | $^{3'}$AGA$^{N}$GGA$^{N}$GGAAG-GGAGG$^{5'}$-$^{5'}$CTTCCTCCTCT$^{3'}$ | 54 |

These embodiments of nucleotide loops created more stability of the triplex than the hexaethyleneglycol linker. Best results were obtained with the reversed TTTT linker (hairpin B-22ALT1, an increased on Tm of 6 degrees), followed by the TTTT linker (hairpin B-22ALT2, ΔTm 4 degrees) and the GGAGG linker (hairpin B-22ALGA, ΔTm 3 degrees).

Binding of hairpins to target were also analysed by gel-shift experiments. In these experiments the target was labelled radioactively with [γ-$^{32}$P]-ATP and polynucleotide kinase and increasing amounts of the hairpins were added to a solution of the labelled target. After incubation at room temperature for 30 min-1 hr

the mixtures were analysed by polyacrylamide gel electrophoresis. The formation of the triplex was followed by the appearance of a radioactive band with less mobility than the band corresponding to the target alone. One experiment used the binding of hairpin R-22G to target WC-11mer and to a double-stranded target consisting of an equimolar mixture of target WC-11mer ($^{5'}$TCTCCTCCTTC$^{3'}$) and its complementary purine strand ($^{3'}$AGAGGAGGAAG$^{5'}$) at pH 7.0. When the labelled oligonucleotide is the target pyrimidine strand (WC-11mer) the appearance of a new radioactive band with lower mobility is observed in both single and double-stranded targets indicating the formation of the triplex. On the contrary when the labelled oligonucleotide is the purine strand no new band is observed indicating that the binding of hairpins only occurred to the target pyrimidine strand.

**Methods of Use of Hairpins**

The present invention further comprises compositions and methods for the detection of nucleic acid sequences. These methods include automation procedures for rapid screening and diagnostic applications. One embodiment of the present invention is the use of one or more hairpin structures that hybridize to a nucleic acids to form triplex structures. The present invention also comprises hybridization of multiple hairpins that, for example, can distort the structure of the nucleic acid and allow for interactions such as intercalating agents, binding of other probes or restriction of the unbound or distorted areas.

In a preferred method, two hairpin probes are hybridized to one strand of the double-stranded DNA so that the probes flank the target sequence on both sides and form two triplex structures. These two triplex structures force the ds DNA to "breathe" open between the triplex structures and allow for binding of other nucleotide structures or probes within this region, if required by a particular method.

See Figure 21 A and B, which illustrates an embodiment of the triplex formed by addition of self-annealing probes to ds DNA. Two hairpin structures are used to form two triplex regions, and one of the hairpin structures is used as a capture molecule, in which the loop portion has a peptide attached that can be bound by a specific antibody. The capture molecule is a magnetic bead attached to the loop portion of the hairpin structure and the reporter probe is detected using a monoclonal antibody that is recognized by a second antibody that is labeled using a liposome filled with a fluorophore. The entire reacted structure is detected using an antibody that is labeled using a liposome filled with a fluorophore.

A preferred embodiment comprises methods and compositions involving hybridization of at least one hairpin probe that comprises a 5' to 3' purine sequence, followed by a loop sequence, followed by a 3' to 5' pyrimidine sequence that is complementary to the purine sequence, as shown in Figure 3. The loop sequence comprises any sequence that will not hybridize to itself. The loop may be of any sequence or include any number of nucleotides, preferably four thymidine resides, or a six-member ethylene glycol chain. The ethylene glycol chain can be derivatized to allow for attachment of different molecules. Preferably, the purine and pyrimidine sequences are approximately eleven nucleotides each. The pyrimidine sequence may be synthesized using reverse phosphoramidites.

In some embodiments, at least one of the hairpin probes is labeled with a peptide, preferably a hexapeptide, comprising a known amino acid sequence. Antibodies, that are specific for this amino acid sequence, are attached to magnetic beads. The binding of the antibody and the peptide binding partner allows for the capture of the DNA target comprising the triplex structures. In another embodiment, the loop structure of the hairpin probe is attached directly to a magnetic bead for capture. The antibodies used may be monoclonal, polyclonal or antibody fragments.

Additionally, other specific binding partner pairs, such as those known in the art, can be used with the present invention.

Once captured, the DNA target can be detected by any of the numerous detection systems known to those skilled in the art. A novel detection system comprises use of a single-stranded reporter probe that binds between the two triplex formations, as shown in Figure 21A and B. The reporter comprises a nucleic acid sequence that has a peptide attached to it that can be detected by a first antibody. A second antibody, specific for the first antibody, is attached to a liposome filled with a fluorophore. Upon addition of the complement cascade proteins, the liposome could be lysed and the fluorophore released and thus, detected.

In another preferred embodiment, the detection step may comprise binding of a single-stranded reporter probe having a peptide, preferably a hexapeptide, attached. The presence of the peptide is detected using an antibody to the peptide that is complexed with a liposome. The liposome is filled with a chosen fluorophore and modified at a very low and limited number of sites with the antibody specific for the peptide of the reporter probe.

The liposomes of the present invention may comprise different substrates and the outer surfaces may be modified using methods known to those skilled in the art, such as modification methods involving, but not limited to, thiol groups or amino groups. Monoclonal antibodies or polyclonal antibodies or antibody fragments could be attached to such liposomes. Preferably, four to five antibody molecules per liposome are used.

Any liposome that can be filled with a detectable agent can be used in the present invention. Depending on the nature of the substrate of the liposome, the liposome can be filled with the detectable agent using a variety of methods. One method is passive diffusion. For example, a liposome of approximately 50 nm in

diameter will take up 30 to 40 molecules of carboxy-fluorescein. Larger liposomes, such as those having diameters of approximately 150 nm, can take up more detectable agent molecules. With one reporter probe, there would be a minimum of 30-fold signal amplification with 50 nm liposomes, over the use of a single detectable molecule attached to a reporter probe.

Once the capture step and all the wash steps are completed, it is not critical that the bound structures are kept intact. At that point, the liposomes could be lysed by any method to release the detectable agents. For example, mild conditions, such as those using ionic detergents could be used to open the liposome channels and release the detectable agent.

The present invention also comprises multiplexing. Such methods and compositions may be used with heterozygous samples and or SNPs by using reporter probes of different sequence specificity and attached to different peptides. Each peptide/probe would be detected by a different antibody attached to a liposome carrying a detectable agent that is not carried by any other liposome. It is preferred that the detectable agents do not interfere with one another.

**Hybridization properties of 8-amino-2'-deoxyinosine**

In the recent years, interest has focused on base analogues which may form base pairs with the four natural bases. 2'-deoxyinosine is one of the most successfully used. See references 44-49. Due to the structural similarity of 8-amino-2'-deoxyinosine to 2'-deoxyinosine, the hybridisation properties of 8-amino-2'-deoxyinosine were measured spectrophotometrically. Pentadecanucleotide duplexes carrying at the central position all possible base pairs between 8-aminohypoxanthine ($I^N$) or hypoxanthine (I) with the four natural bases were prepared. The melting temperatures of the transition duplex to coil of duplexes containing 8-amino-2'-

deoxyinosine or 2'-deoxyinosine opposite the four natural bases are shown in Table 9.

Table 9. Melting temperatures (°C) of duplexes carrying 8-amino-hypoxanthine ($I^N$) and hypoxanthine (I) measured at 0.15 M NaCl, 50 mM Tris-HCl buffer pH 7.5.

5' TAGAGGXTCCATTGC 3'

3' ATCTCCYAGGTAACG 5'

| | X = $I^N$ | X = I |
|---|---|---|
| Y = C | 56 | 58 |
| Y = A | 53 | 55 |
| Y = G | 54 | 53 |
| Y = T | 53 | 52 |

Melting temperatures of duplexes carrying 8-aminohypoxanthine were between 53-56 °C (3 degrees of difference) while duplexes carrying hypoxanthine were between 52-58 °C (6 degrees of difference) indicating that 8-amino-2'-deoxyinosine is a good alternative to 2''deoxyinosine as universal base at ambiguous positions in DNA primers and probes.

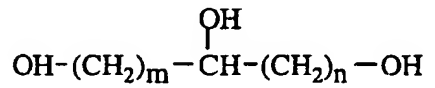
The present invention also comprises compositions and methods of making parallel stranded oligonucleotides. Such methods comprise use of spacer arms and oligonucleotides that may comprise any number of bases. The synthesis of 3'-3' or 5'-5' attached oligonucleotides is described herein. The oligonucleotides may have the same or different sequences of nucleotides. Oligonucleotide synthesis is usually carried out from the 3' to the 5' terminus. However, in some cases it is necessary to synthesize oligonucleotides in the opposite sense (5' to 3'). Modifying the terminal linkages from the natural 3'-5' to 3'-3' and/or 5'-5' results in an increased

resistance in those oligonucleotides against exonuclease activity, especially 3'-exonuclease activity. A general scheme for making the linkers is shown in Figure 7.

Synthesis of the spacer arm: 1.3 bis (dimethoxytrityl)propane-1,2,3-triol.

Glycerol was used as starting material for the preparation of this spacer arm, but any compound with the structure shown below can be used with good results.

Structure of Starting Material for Spacer Arms



The procedure to prepare the glycerol-spacer arm (S)* is shown in Figure 8. (S*-symmetrical). The glycerol spacer arm was attached to the LCAA-CPG using the oxalyl linker. The oxalyl can be replaced by succinyl or sarcosyl spacer arms. As shown in Figure 8, i) is the reaction of dimethoxytrityl chloride in pyridine, ii) are the reactions a) oxalyl chloride and 1,2,4-triazole in acetonitrile, b) LCAA-CPG (long chain alkylamino-controlled pore glass); c) dimethylaminopyridine (DMAP), acetic anhydride, THF (tetrahydrofuran).

The first step of the synthesis consisted of the selective protection of the primary hydroxyl function. The protected product was dissolved in acetonitrile/pyridine (2:1) and then added to a previously prepared mixture of oxalyl chloride and 1,2,4-triazole in anhydrous acetonitrile. After 1 hour the solution was transferred to a gastight syringe containing the LCAA-CPG and the reaction was allowed to proceed for 30 minutes. The liquid was ejected from the syringe and the solid support washed successively with acetonitrile, anhydrous methanol (to cap residual oxalyl triazolide groups) and acetonitrile.

To acetylate underivatized amino groups from further reaction the solid support was treated with an equivolume mixture of tetrahydrofuran (THF) solutions of DMPA and acetic anhydride for 30 minutes. At the end, the support was washed with pyridine, acetonitrile and diethyl ether, air dried and then dried *in vacuo*.

Synthesis of the 3'-3' and 5'-5' attached oligonucleotides with identical sequences.

3'-3' and 5'-5' attached oligonucleotides with identical sequences were prepared on an automatic DNA synthesizer using the glycerol spacer arm (S)-LCAA-CPG and standard or reversed 2-cyanoethyl phosphoramidite chemistry (Figure 9A & B). Oligonucleotides were synthesized with the last DMT group on to help reverse-phase HPLC-purification. After the assembly of the sequences, the oligonucleotides were cleaved from the support by a treatment of 32% aqueous ammonia at 55°C for 16 hours. Ammonia solutions were then concentrated to dryness and the products purified using the standard protocols for reverse-phase HPLC.

**Synthesis of 3'-3' or 5'-5' attached oligonucleotides with different sequences**

Synthesis of the spacer arm: 1-Dimethoxytrityl-3-laevulinyl pronane-1,2,3-triol.

In this case, glycerol was also used as starting material for the preparation of the spacer arm. Any compound with the structure shown above can be used with good results.

The procedures to prepare the glycerol spacer arm (As)* is shown in Figure 10. (AS*-Asymmetrical.) The synthesis of the glycerol-spacer arm (AS) was attached to the oxalyl linker, though the oxalyl can be replaced by succinyl or sarcosyl spacer arms. The reaction steps shown in Figure 10 comprise reactions of i) dimethoxytrityl chloride in pyridine; ii) laevulinic anhydride and

dimethylaminopyridine in dichloromethane; iii) oxalylchlroide and 1,2,4-triazol in acetonitrile, b) LCAA-CPG, c) DMAP, acetic anhydride, THF.

The first step of the synthesis consisted of the selective protection of one of the primary hydroxyl functions. The reaction was carried out in pyridine using an excess of glycerol.

The monoprotected product was dissolved in anhydrous dichloromethane. 4-Dimethylaminopyridine, triethylamine and laevulinic anhydride were added to the stirred solution with exclusion of moisture, and the reaction mixture was left at room temperature overnight. After the work-up, the desired compounds were purified by column chromatography on silica gel.

The 1,3-protected glycerol was dissolved in acetonitrile-pyridine (2:1) and then added to a previously prepared mixture of oxalyl chloride and 1,2,4-triazole in anhydrous acetonitrile. After 1 hour the solution was transferred to a gas-tight syringe containing the LCAA-CPG and the reaction is allowed to proceed for 30 minutes. The liquid was ejected from the syringe and the solid support washed successively with acetonitrile, anhydrous methanol (to cap residual oxalyl triazolide groups) and acetonitrile.

To acetylate underivatized amino groups from further reaction, the solid support was treated with an equivolume mixture of tetrahydrofuran (THF) solutions of DMAP and acetic anhydride for 30 minutes.

At the end, the support was washed with pyridine, acetonitrile, and diethyl ether, air dried and then dried *in vacuo*.

Synthesis of the 3'-3' and 5'-5' attached oligonucleotides with different sequences.

3'-3' and 5'-5' attached oligonucleotides with different sequences can be synthesized on an automatic DNA synthesizer using the glycerol spacer arm (As)-

LCAA-CPG and standard or reversed 2-cyanoethyl phosphoramidite chemistry (Figure 11A and B).

At the beginning of the synthesis, the dimethoxytrityl-protecting group from the spacer arm was removed, allowing chain extension is that direction to complete the desired sequence. Then, the dimethoxytrityl protecting group of the last monomer added was removed and the resultant hydroxyl group was capped by acetylation to prevent further chain extension from this part. In order to be able to chain extend from the other hydroxyl group of the glycerol-spacer arm (As), the laevulinyl group was cleaved with hydrazine hydrate in pyridine acetic acid. Chain extension to give the desired 3'-3' or 5'-5' attached oligonucleotides was continued with the same type of phosphoramidites used for assembling the first chain. In this case, the dimethoxytrityl group of the last monomer added has to be left on the help reverse-phase HPLC-purification.

After the assembly of the sequence, the oligonucleotides were cleaved from the support by a treatment of 32% aqueous ammonia at 55°C for 16 hours. Ammonia solutions were then concentrated to dryness and the products purified using the standard protocols for reverse-phase HPLC.

Modified Nucleosides.

The present invention further comprises compositions and methods for the preparation of nucleosides with one or more linkers. For example, pyrimidine nucleosides with linker arms at $N^4$ of 2'-deoxycytidine using the 1,2-bis(aminoethoxy)ethane linker arm are disclosed herein, but any compound with structure shown below can be successfully used.

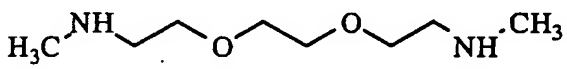
Structures for Preparing Pyrimidine

Nucleosides with Linker Arms

1.

2.

3.



The compounds 1 or 2 can be purchased from commercially available sources. The spacer 3 is synthesized by the reaction of 1,2-bis(2-chloroethoxy)ethane with a 40% water solution of methylamine in a pressure vessel at 160°C for 7 days.

Basic approaches for attaching alkyl linkers to $N^4$.

The three basic approaches for attaching alkyl linkers to $N^4$ have been:

1. Introductions of a leaving group such as triazolyl or nitrophenyl at $O^4$ of the 2'-deoxyuridine or deoxythymidine followed by a reaction with a diaminoalkane. See references 28-30.
2. Bisulphate catalyzed transamination of 2'-deoxycytidene with a diaminoalkane. See reference 31.

3. Displacement of sulphur from 4-thiopyrmidines with a diaminoalkane. See reference 32.

In the synthesis methods, the first approach was to synthesize the intermediates needed (see Figure 12). The first intermediate synthesized was 3',5'-O-bis(trimethylsilyl)-2'-deoxy-$O^4$-(2-nitrophenyl)uridine. Following the steps of Figure 12, the first reaction is i), 1,1,1,3,3,3,-hexamethyldisilazane in DMF; ii) a) 0 triethylamine, 2-mesitylenesulphonyl chloride and 4-dimethylaminopyridine in dichloromethane, b) 1,4-diazabicyclo(2.2.2) octane and 2-nitrophenol in dichloromethane. It is contemplated by the present invention that other methods may be used to obtain the intermediates.

Attachment of protected fluorescein to the spacer arm.

A preferred method of the present invention is the use of protected fluorescein isothiocyanate (supplied by Molecular Probes) in order to avoid modifications of the fluorophore during the oligonucleotide synthesis. The compound is designed to produce, on deprotection and isolation of the derived oligonucleotide, the same fluorescein isothiocyanate. See reference 26. The reactions of fluorescein isothiocyanate with primary amines are well described in the literature. The structure of the protected fluorescein attached to the spacer arm is shown in Figure 13.

Synthesis of the 2'-Deoxy-5'-O-dimethoxytrityl-$N^4$-[8-amino-$N^8$-(3',6'-dipivaloylfluoresceinyl)-3,6-dioxaoctyl]cytidine-3'-O-(2-cyanoethyl-*N,N*-diisopropyl phosphoramidite).

The attachment of the spacer arm bearing the protected fluorescein to the intermediate nucleoside and the synthesis of the final phosphoramidite are shown in the Figure 14A & B. The synthesis of the $N^4$-spacer arm-fluorescein labelled-2'-

deoxycytidine phosphoramidite included the reactions of i) ethyldiisopropylaminer, dimethylformamide, overnight at room temperature; ii) dimethoxytrityl chloride in pyridine; iii) 2-cynoethoxy-N,N-diisopropylaminochloro phosphine and diisopropylethylamine in dichloromethane. The displacement reaction on the 4 position proceeded rather slowly, generally overnight, but with very good yields. The next steps were the established protocols for the protection of the 5'-hydroxyl function followed by the phosphitylation reaction with chloro-(2-cyanoethoxy)diisopropylaminophosphine.

Attachment of 5-bromouracil (cleavage agent) to the spacer arm.

The attachment of 5-bromouracil to the spacer arm is a two step procedure (see Figure 15). The attachment of the 5-bromouracil to the 1,2-bis(aminoethoxy)ethane linker arm comprised the reactions of i) bromoacetic acid, KOH, and water; and ii) 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOOBt), N,N'-diisopropylcarbodiimide (DIPC), and N-etilmorfoline (NEM) in DMF.

The first reaction was done to produce 1-carboxymethyl-5-bromouracil. Due to the structural similarity of 5-bromouracil with thymine, a procedure similar to the one that has been developed to prepare the 1-carboxymethylthymine was used. See reference 33. The second step was the attachment of the 1-carboxymethyl-5-bromouracil to the 1,2-bis(aminoethoxy)ethane linker arm.

Synthesis of the 2'Deoxy-5'-O-dimethoxytrityl-$N^4$-{8-amino-$N^8$-[[(5-bromo)uracil-1-yl]acetyl]-3,6dioxaoctyl}cytidine-3'-O-(2-cyanoethyl-*N,N*-diisopropyl phosphoramidite).

The attachment of the spacer arm bearing the 5-bromouracil to the intermediate nucleotide and the synthesis of the final phosphoramidite were carried out in the same way as for the fluorescein analogue (Figure 16A & B). Synthesis of the $N^4$-spacer arm-5-bromouracil-2'-deoxycytidine phosphoramidite included the reactions of i) ethyldiisopropylamine, dimethylformamamide, overnight at room temperature; ii) dimethoxytrityl chloride in pyridine, iii) 2-cyanoethoxy-N,N-diisopropylaminochloro phosphine and diisopropylethylamine in dichloromethane.

The displacement reaction on the 4 position proceeded rather slowly (overnight) but with very good yields. The next steps were the established protocols for the protection of the 5'-hydroxyl function, followed by the phosphitylation reaction the chloro-(2-cyanoethoxy)diisopropylaminophosphine.

The present invention also comprises compositions and methods of synthesis and use of modified nucleosides. Particularly, the synthesis of reversed phosphoramidites of 5-methyl-2'-deoxycytidine, 8-amino-2'deoxyadenosine, 8-amino-2'-deoxyguanosine and 8-amino-2'-deoxyhyphoxanthine (8-amino-2'-deoxyinosine). These compounds can be used for the incorporation of triplex stabilizing nucleotides for assembling oligonucleotides in the 5' to 3' sense. The use of a pixyl moiety as a protecting group for the 3' hydroxyl improved the yield of the product.

Synthesis of the 8-amino-2'-deoxy-$N^6$,$N^8$-bis(dimethylamino-methyliden)-3'-O-(9-phenylxanthen-9-yl)adenosine 5'-O-(2cyanoethyl-*N*,*N*-diisoproplyphosphoramidite.

The synthesis of the 8-amino-2'-deoxyadenosine reversed monomer is illustrated in Figure 17A, B & C. Synthesis of the 8-amino-2'-deoxyadenosine reversed monomer, comprised the following: i) Bromine solution in 1M sodium acetate (pH 5.4), ii) sodium azide in DMF, 75°C, overnight, iii) Pd/C, $H_2$ in water-ethanol (6:4), iv) N, N-dimethylformamide dimethyl acetal in DMF, v) tert-butyldiphenylchlorosilane and imidazole in DMF, vi) 9-phenylxanthen-9-yl (Pixyl) chloride in pyridine, vii) tetrabutylammonium flouride (TBAF) in THF, viii) 2-cyanoethoxy-N,N-diisopropylaminochloro phosphine and diisopropylethylamine in dichloromethane. The 8-amino-2'-deoxy-$N^6$,$N^8$-bis-(dimethyl-aminomethyliden)adenosine was prepared as previously described. See references 4,33 and 36. In order to introduce the acid labile 9-phenylxanthen-9-yl (Pixyl) group on the 3'-hydroxy moiety it was necessary first to selectively protect the 5'-hydroxy function. See reference 37. The bulky lipophilic *tert*-butyldiphenylsilyl group carried out this protection. Overnight reaction of the free 3'-hydroxyl group with pixyl chloride pyridine produced the 3'-pixyl protected nucleoside. This protection can be alternatively done by using dimethoxytrityl chloride, which produced poorer yields. Removal of the *tert*-butyldiphenylsilyl protecting group with tetrabutylammonium fluoride (TBAF) allowed the isolation of the 3'-pixyl compound. Finally, 5'-hydroxy group phosphilylation with chloro-(2-cyanoethoxy)diisopropylaminophosphine afforded the desired monomer.

Synthesis of the 8-amino-2'-deoxy-$N^2$,$N^6$-bis(dimethyl-aminomethyliden)-3'-O-(9-phenlxanthen-9-yl)guanosine-5'-O-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite).

The synthesis of the 8-amino-2'-deoxyguanosine reversed monomer is illustrated in Figure 18A & B. Synthesis of the 8-amino-2'-deoxyguanosine reversed monomer comprised the following steps: i) N,N-dimethylformamide dimethyl acetal in DMF, ii) tert-butyldiphenylchlorosilane and imidazole in DMF, iii) 9-phenylxanthen-9-yl (Pixyl) chloride in pyridine, iv) tetrabutylammonium fluoride (TBAF) in THF, v) 2-cyanoethoxy-N,N-diisopropylaminochloro phosphine and diisopropylethylamine in dichloromethane. The 8-amino-2'-deoxy-$N^6$,$N^8$-bis(dimethyl-aminomethyliden)guanosine was prepared as previously described. See reference 12. In order to introduce the acid labile 9-phenylxanthen-9-yl (Pixyl) group on the 3'-hydroxy moiety it is necessary first to selectively protect the 5'-hydroxy function. The bulky lipophilic *tert*-butyldiphenylsilyl group carried out this protection. Overnight reaction of the free 3'-hydroxyl group with pixyl chloride pyridine produced the 3'-pixyl protected nucleoside. This protection can be done alternatively by using dimethoxytrityl chloride, which produces poorer yields. Removal of the *tert*-butyldiphenylsilyl protecting group with tetrabutylammonium fluoride (TBAF) allowed the isolation of the 3'-pixyl compound. Finally, the 5'-hydroxy group phosphitylation with chloro-(2-cyanoethoxy)diisopropylaminophosphine afforded the desired monomer.

Synthesis of the 8-amino-2'-deoxy-$N^8$-isobutyryl-3'-O-(9-phenylxanthen-9-yl)inosine-5'-O-(2-cyanoethyl-*N*, *N*-diisopropyl-phosphoramidite).

In summary, the synthesis comprised reactions comprising the following steps, i) Adenosine deaminase in a mixture of dimethylsuphoxide and 0.1M aqueous sodium phosphate (1:3), ii) 1,1,1,3,3,3-Hexamethyldisilazane in DMF, iii)

Isobutyryl chloride in pyridine, iv) a) Dioxane/Methanol/25% aqueous ammonia (1:1:2), b) tert-butyldiphenylchlorosilane and imidazole in DMF, v) 9-phenylxanthen-9-yl (Pixyl) chloride in pyridine, vi) a) tetrabutylammonium fluoride (TBAF) in THF, b) 2-cyanoethoxy-N, N-diisopropylaminochloro phosphine and diisopropylethylamine in dichloromethane. The 8-amino-2'-deoxyinosine was obtained from the treatment of 8-amino-2'deoxyadenosine with adenosine deaminase at 37°C for 72 hours. The exocyclic amino protection was then introduced via transient protection procedure to give the $N^8$-isobutyryl compound. In order to introduce the acid labile 9-phenylxanthen-9-yl (Pixyl) group on the 3'-hydroxy moiety it was necessary first to selectively protect the 5'-hydroxy function. The bulky lipophilic *tert*-butyldiphenylsilyl group carried out the protection. Overnight reaction of the free 3'-hydroxyl group with pixyl chloride pyridine produced the 3'-pixyl protected nucleoside. This protection can be done alternatively by using dimethoxytrityl chloride, which produces poorer yields. Removal of the *tert*-butyldiphenylsilyl protecting group with tetrabutylammonium fluoride (TBAF) allowed the isolation of the 3'-pixyl compound. Finally, 5'-hydroxy group phosphitylation with chloro-(2-cyanothoxy) diisopropylaminophosphine afforded the desired monomer. The synthesis of the 8-amino-2'deoxyinosine reversed monomer is illustrated in Figure 19A & B.

<u>Synthesis of 2'-deoxy-$N^4$-isobutyryl-5-methyl 3'-O-(9-phenylxanthen-9-yl)cytidine-5'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite)</u>.

The reactions comprise the following steps i) a) 1,1,1,3,3,3-Hexamethyldisilazane in DMF, b) Isobutyryl chloride in pyridine, c) Dioxane/Methanol/25% aqueous ammonia (1:1:2), ii) tert-butyldiphenylchlorosilane and imidazole in DMF, iii) 9-phenylxanthen-9-yl (Pixyl) chloride in pyridine, iv)

tetrabutylammonium fluoride (TBAF) in THF, v) 2-cyanoethoxy-N,N-diisopropylaminochloro phosphine and diisopropylethylamine in dichloromethane. The procedures to synthesize 5-methyl-2'deoxycytidine are well established. See references 28-30 and 38. The exocyclic amino protection was then introduced via transient protection procedure to give the $N^4$-isobutyryl compound. In order to introduce the acid labile 9-phenylxanthen-9-yl (Pixyl) group on the 3'-hydroxy moiety it was necessary to selectively protect the 5'-hydroxy function. The bulky lipophilic *tert*-butyldiphenylsilyl group carried out the protection. Overnight reaction of the free 3'-hydroxyl group with pixyl chloride pyridine produced the 3'-pixyl protected nucleoside. This protection can be done alternatively by using dimethoxytrityl chloride, which produces poorer yields. Removal of the *tert*-butyldiphenylsilyl protecting group with tetrabutylammonium fluoride (TBAF) allowed the isolation of the 3'-pixyl compound. Finally, 5'-hydroxy group phosphitylation with chloro-(2-cyanothoxy)diisopropylaminophosphine afforded the desired monomer. The synthesis of the 5-methyl-2'-deoxycytidine reversed monomer is illustrated in Figure 20 A & B.

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This invention is illustrated by the included examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other

embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the included claims.

**Example 1: Oligonucleotide Synthesis**

Oligonucleotides were prepared on an automatic DNA synthesizer using standard and reversed 2-cyanoethyl phosphoramidites and the modified phosphoramidites of the 8-aminopurines prepared as described. See references 4, 5, 10-13. The phosphoramidite of the hexaethyleneglycol linker was also obtained from commercial sources. Complementary oligonucleotides containing natural bases were also prepared using commercially available chemicals and following standard protocols. After the assembly of the sequences, oligonucleotide-supports were treated with 32% aqueous ammonia at 55°C for 16 hours. Ammonia solutions were concentrated to dryness and the products were purified by reverse-phase HPLC. Oligonucleotides were synthesized on 0.2μmol scale and with the last DMT group at the 5' end (DMT on protocol) to help reverse-phase purification. All purified products presented a major peak which was collected and analyzed by snake venom phosphodiesterase and alkaline phosphatase digestion followed by HPLC analysis of the nucleosides (HPLC conditions B). Yield (OD units at 260 nm after HPLC purification, 0.2 μmol) were between 6-10 OD.

**HPLC Conditions**

HPLC solutions were as follows. Solvent A: 5% ACN in 100 mM triethylammonium acetate (pH 6.5) and solvent B: 70% ACN in 100mM triethylammonium acetate pH 6.5. For analytical runs the following conditions were used. Column: Nucleosil 1210$C_{18}$, 250 x 4 mm, flow rate: 1 ml/min. Conditions A) a

40 min linear gradient from 0 to 75%B. Conditions B) a 20 min linear gradient from 0 to 20% B. For preparative runs the following conditions were used: Columns: PRP-1 (Hamilton), 250 x 10 mm. Flow rate: 3ml/min. A 30 min linear gradient from 10-80% B (DMT on), or a 30 min linear gradient from 0-50% B (DMT off).

**Melting Experiment**

Melting experiments with triple helix were performed as follows: Solutions of equimolar amounts of the modified oligonucleotide (22-mer) and the target Watson-Crick pyrimidine strand (11-mer) were mixed in the appropriate buffer. The solutions were heated to 90°C, allowed to cool slowly to room temperature and then samples were kept in the refrigerator overnight. UV absorption spectra and melting experiments (absorbance vs. temperature) were recorded in 1 cm path-length cells using a spectrophotometer, which has a temperature controller with a programmed temperature increase of 0.5°C/min. Melts were run on duplex concentration of 4 $\mu$M at 260 nm.

**Example 2**

Preparation of 8-amino-2'-deoxyinosine

In a round-bottom flask 2 g (7.5 mmol) of 8-amino-2'-deoxyadenosine (**Long R.A. et al *J. Org. Chem.* (1967) 32:2751-2756**) were dissolved with 150 ml of 0.1 M aqueous sodium phosphate buffer (pH 7.5). Adenosine deaminase (150 mg of crude powder from calf intestinal mucosa) was added. The resulting solution was stirred at 37 °C for 72 hrs. A precipitate was formed that was the desired product. Completion of the reaction was checked by TLC (ethanol / dichloromethane 1:4). It was observed the disappearance of the starting material to produce a more polar compound. The precipitate was filtered yielding 1.8 g (6.7 mmol, 89% yield) of 8-amino-2'-deoxyinosine.

**Example 3**

Preparation of 8-amino-2'-deoxy-5'-O-dimethoxytrityl-$N^8$-isobutyryl-inosine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite

The nucleoside from Example 2 (1.5 g, 5.6 mmol) was dried by evaporating dry pyridine (10 ml) twice. The resulting residue was dissolved in 20 ml of dry pyridine and treated with phenoxyacetic anhydride (24 mmol, 7.2 g). After 2 hrs of stirring at room temperature water (3 ml) was added to destroy the excess of anhydride and the mixture was concentrated to dryness. A mixture of triethylamine-pyridine-water 20/20/60 was added to deprotect the 3' and 5'-hydroxy functions. After 15 min of stirring at room temperature, the solution was concentrated to dryness. The residue was treated with dichloromethane and the product was not soluble. The resulting precipitate was dissolved in 20 ml of pyridine and was treated with trimethylchlorosilane (20 mmol, 2.9 ml). After 25 min of magnetic stirring at

room temperature, isobutyryl chloride (16.8 mmol, 1.8 ml) was added and the solution was stirred for 3 hr at room temperature. The mixture was cooled with an ice-bath and water ( 2 ml) was added followed by 2 ml of concentrated ammonia. After 15 min of magnetic stirring at room temperature, the solution was concentrated to dryness. The resulting product was purified by silica gel chromatography yielding 1.8 g of $N^8$-isobutyryl-$N^8$-phenoxyacetyl-8-amino-2'-deoxyinosine (4 mmol, 71 % yield).

The product described above was dissolved into dry pyridine (20 ml) and dimethoxytrityl chloride (1.7 g, 5.2 mmol) was added to the solution. After 3 hr of magnetic stirring at room temperature, methanol (2 ml ) was added and the solution was concentrated to dryness. The residue was dissolved in dichloromethane and the solution was washed with aqueous sodium bicarbonate solution and brine. The organic phase was dried over $Na_2SO_4$ and evaporated. Silica gel chromatography yielded 2.1 g (3.4 mmol, 85% yield) of 5'-dimethoxytrityl- $N^8$-isobutyryl-8-amino-2'-deoxyinosine. Unexpectedly, the phenoxyacetyl group was eliminated during the work-up due to the lability of this group.

The protected nucleoside (3.4 mmol) described above was dissolved in dry dichloromethane (15 ml) and diisopropylethylamine was added (2.4 ml, 13.7 mmol). To the solution chloro 2-cyanoethoxy diisopropylamino phosphine (1.1 ml, 5,1 mmol) was added dropwise with a syringe. After 1 hr of magnetic stirring at room temperature, methanol (2 ml) was added and the solution was concentrated to dryness. The resulting residue was dissolved in dichloromethane and washed with aqueous sodium bicarbonate solution and brine. The organic phase was dried over $Na_2SO_4$ and evaporated. Silica gel chromatography yielded 2.1 g (2.6 mmol, 76% yield) of the desired phosphoramidite.

**Example 4**

Preparation of Oligomers Containing 8-aminopurines

Oligonucleotides were prepared on an automatic DNA synthesizer using standard and reversed 2-cyanoethyl phosphoramidites and the modified phosphoramidites of the 8-aminopurines. The phosphoramidite of protected 8-amino-2'-deoxyinosine was dissolved in dry dichloromethane to make a 0.1 M solution. The rest of the phosphoramidites were dissolved in dry acetonitrile (0.1 M solution). The phosphoramidite of the hexaethyleneglycol linker was obtained from commercial sources. Complementary oligonucleotides containing natural bases were also prepared using commercially available chemicals and following standard protocols. After the assembly of the sequences, oligonucleotide-supports were treated with 32% aqueous ammonia at 55 °C for 16 h except for oligonucleotides having 8-aminoguanine. In this case a 0.1 M 2-mercaptoethanol solution in 32% aqueous ammonia was used and the treatment was extended to 24 h at 55 °C. Ammonia solutions were concentrated to dryness and the products were purified by reverse-phase HPLC. Oligonucleotides were synthesized on 0.2 μmol scale and with the last DMT group at the 5' end (DMT on protocol) to help reverse-phase purification. All purified products presented a major peak which was collected and analyzed by snake venom phosphodiesterase and alkaline phosphatase digestion followed by HPLC analysis of the nucleosides (HPLC conditions B). Yield (OD units at 260 nm after HPLC purification, 0.2 μmol) were between 6-10 OD. HPLC conditions : HPLC solutions are as follows. Solvent A: 5% ACN in 100 mM triethylammonium acetate (pH 6.5) and solvent B: 70% ACN in 100 mM triethylammonium acetate pH 6.5. For analytical runs the following conditions were used. Column: Nucleosil 120$C_{18}$, 250 x 4 mm, flow rate: 1 ml/min. Conditions A) a 40 min linear gradient from 0 to 75%B. Conditions B) a 20 min linear gradient from 0 to 20% B. For preparative runs the

following conditions were used: Columns: PRP-1 (Hamilton), 250 x 10 mm. Flow rate: 3 ml/min. A 30 min linear gradient from 10-80% B (DMT on), or a 30 min linear gradient from 0-50% B (DMT off).

**Example 5**

Binding of oligomers of the invention to target sequences by melting experiments

Melting experiments with triple helix were performed as follows: Solutions of equimolar amounts of the modified oligonucleotide (22-mer) and the target Watson-Crick pyrimidine strand (11-mer) were mixed in the appropriate buffer. The solutions were heated to 90 °C, allowed to cool slowly to room temperature and then samples were kept in the refrigerator overnight. UV absorption spectra and melting experiments (absorbance vs temperature) were recorded in 1 cm path-length cells using a spectrophotometer, which has a temperature controller with a programmed temperature increase of 0.5 °C/min. Melts were run on duplex concentration of 4 μM at 260 nm.

**Example 6**

Binding of oligomers of the invention to target sequences by gel-shift experiments.

In addition to melting experiments the binding of oligomers of the invention to their polypyrimidine targets was analysed by gel retardation assays. The following targets were studied:

Single stranded target:

WC-11mer : $^{5'}$**TCTCCTCCTTC**$^{3'}$ and

Double stranded target:

WC-11mer : $^{5'}$**TCTCCTCCTTC**$^{3'}$

Complementary purine strand$^{3'}$**AGAGGAGGAAG**$^{5'}$

The target was radioactively labelled at the 5' end by T4 polynucleotide kinase and [$\gamma$-$^{32}$P]-ATP. The reaction was performed with 35-50 µmol of the target oligonucleotide dissolved in 20 µl of kinase buffer. After incubation at 37°C for 45 min, the solution was heated at 70 °C for 10 min to denature the enzyme and the solution was cooled to room temperature. To the solution 60 µl of a 50 mM potassium acetate in ethanol were added and the mixture was left at –20 °C for at least 3 hr. The mixture was centrifuged at 4 °C for 45 min (14000 rpm) and the supernatant was removed. The pellet was washed with 60 µl of 80% ethanol and centrifuge for 20 min at 4 °C. The supernatant was removed and the pellet was dissolved in 0.2 ml of water.

The radiolabelled target was incubated with the hairpins of the invention in 0.1 M sodium phosphate / citric acid buffer of pH ranging from 5.5 to 7.0 at room temperature for 30-60 min. The hairpins were added increasing amounts from 2 to 200 molar equivalents. After incubation the mixtures were analysed by 10-15% polyacrylamide gel electrophoresis. The buffer used on the electrophoresis was the same buffer used during the incubation : 0.1 M sodium phosphate / citric acid buffer of pH ranging from 5.5 to 7.0. The formation of the triplex was followed by the appearance of a radioactive band with less mobility than the band corresponding to the target alone. The sequences of the hairpins are shown in Table 1.

**Example 7**

Hybridization properties of oligonucleotides carrying 8-amino-2'-deoxyinosine

Solutions of equimolar amounts of the pentadecamer carrying 8-amino-2'-deoxyinosine or 2'-deoxyinosine at the central position and its complementary sequences carrying each of the four natural bases opposite to the modified base were

mixed in the appropriate buffer. The solutions were heated to 90 °C, allowed to cool slowly to room temperature and then samples were kept in the refrigerator overnight. UV absorption spectra and melting experiments (absorbance vs temperature) were recorded in 1 cm path-length cells using a spectrophotometer, which has a temperature controller with a programmed temperature increase of 0.5 °C/min. Melts were run on duplex concentration of 4 μM at 260 nm.

**Example 8**

A preferred method of use of the present invention comprises the following steps.

1. Mildly heat the sample DNA at 50 -60° C for approximately 10 minutes.
2. Add a first specific hairpin probe.
3. Incubate at 50° C to allow the formation of the triplex structure.
4. Add a second hairpin probe, covalently attached to the magnetized beads via a spacer arm linked to the loop region of the hairpin.
5. Incubate at approximately 50° C to allow a second triplex structure to form.
6. Add the magnets to concentrate the beads/sample complex.
7. Wash several time while the magnets are present to eliminate the excess unbound first and second hairpins.
8. Remove the magnets and resuspend the bead/sample complex.
9. Add a reporter probe modified with a hexapeptide.
10. Incubate at approximately 50° C to allow the reporter probe to hybridize
11. Add the magnets to concentrate the beads/sample complex

12. Wash several times while the magnets are present to remove any excess, unbound hexapeptide-modified reporter probe

13. Remove the magnets and resuspend the beads/sample complex

14. Add the hexapeptide-specific antibody-modified fluorophore-filled liposomes

15. Incubate at approximately 37° C to allow the antibody-peptide complex to form

16. Add the magnets to concentrate the beads/sample complex

17. Wash several times while the magnets are present to remove any excess, unbound monoclonal antibody-modified fluorophore-filled liposomes

18. Remove the magnets and resuspend the beads/sample complex

19. Add a mild ionic detergent to open the liposome channels and release the fluorophore for detection of the fluorescence.

The disclosures of all patents and publications cited in this application are hereby incorporated by reference in their entireties in order to more fully describe the state of the art to which this invention pertains.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

## Claims

What is claimed is:

1. A triplex-forming oligonucleotide, comprising:

a polypyrimidine sequence connected to a linker, which is connected to a polypurine sequence, wherein the polypurine sequence has at least one modified nucleic acid.

2, The oligonucleotide of Claim 1, wherein the polypyrimidine sequence is in the 5' to 3' orientation.

3. The oligonucleotide of Claim 1, wherein the polypyrimidine sequence is in the 3' to 5' orientation.

4. The oligonucleotide of Claim 1, wherein the polypyrimidine sequence is in the 5' to 3' orientation.

5. The oligonucleotide of Claim 1, wherein the polypurine sequence is in the 5' to 3' orientation.

6. The oligonucleotide of Claim 1, wherein the polypurine sequence is in the 3' to 5' orientation.

7. The oligonucleotide of Claim 1, wherein the one modified nucleic acid is 8-aminoadenine.

8. A method for stabilizing triplex structures, comprising adding an oligonucleotide structure comprising a polypyrimidine sequence linked to a polypurine sequence having at least one modified nucleic acid, to a single stranded nucleic acid sequence, and forming a triplex structure.

9. The oligonucleotide of Claim 8, wherein the polypyrimidine sequence is in the 5' to 3' orientation.

10. The oligonucleotide of Claim 1, wherein the polypyrimidine sequence is in the 3' to 5' orientation.

11. The oligonucleotide of Claim 1, wherein the polypyrimidine sequence is in the 5' to 3' orientation.

12. The oligonucleotide of Claim 1, wherein the polypurine sequence is in the 5' to 3' orientation.

13. The oligonucleotide of Claim 1, wherein the polypurine sequence is in the 3' to 5' orientation.

14. The oligonucleotide of Claim 1, wherein the one modified nucleic acid is 8-aminoadenine.

15. A method for detecting a specific target sequence, comprising, adding at least one oligonucleotide structure comprising a polypyrimidine sequence connected to a linker, which is connected to a polypurine sequence, wherein the polypurine sequence has at least one modified nucleic acid, adding a reporter probe, and detecting the resultant structure.

16. The oligonucleotide of Claim 1, wherein the polypyrimidine sequence is in the 5' to 3' orientation.

17. The oligonucleotide of Claim 1, wherein the polypyrimidine sequence is in the 3' to 5' orientation.

18. The oligonucleotide of Claim 1, wherein the polypyrimidine sequence is in the 5' to 3' orientation.

19. The oligonucleotide of Claim 1, wherein the polypurine sequence is in the 5' to 3' orientation.

20. The oligonucleotide of Claim 1, wherein the polypurine sequence is in the 3' to 5' orientation.

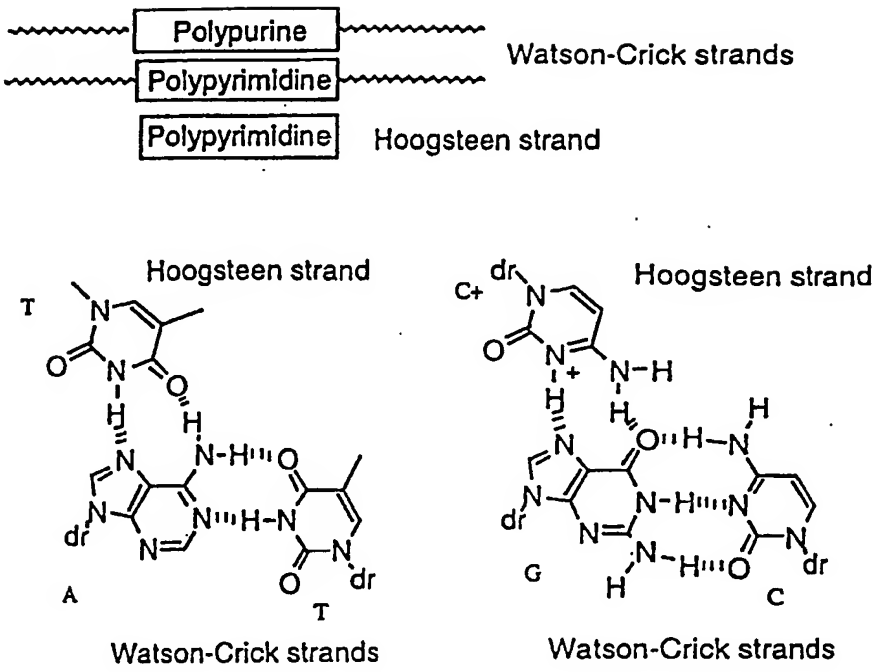

Polypurine
Polypyrimidine
Watson-Crick strands
Polypyrimidine
Hoogsteen strand
Hoogsteen strand
T
A
T
Watson-Crick strands
C+
G
C
Hoogsteen strand
Watson-Crick strands

FIGURE 1

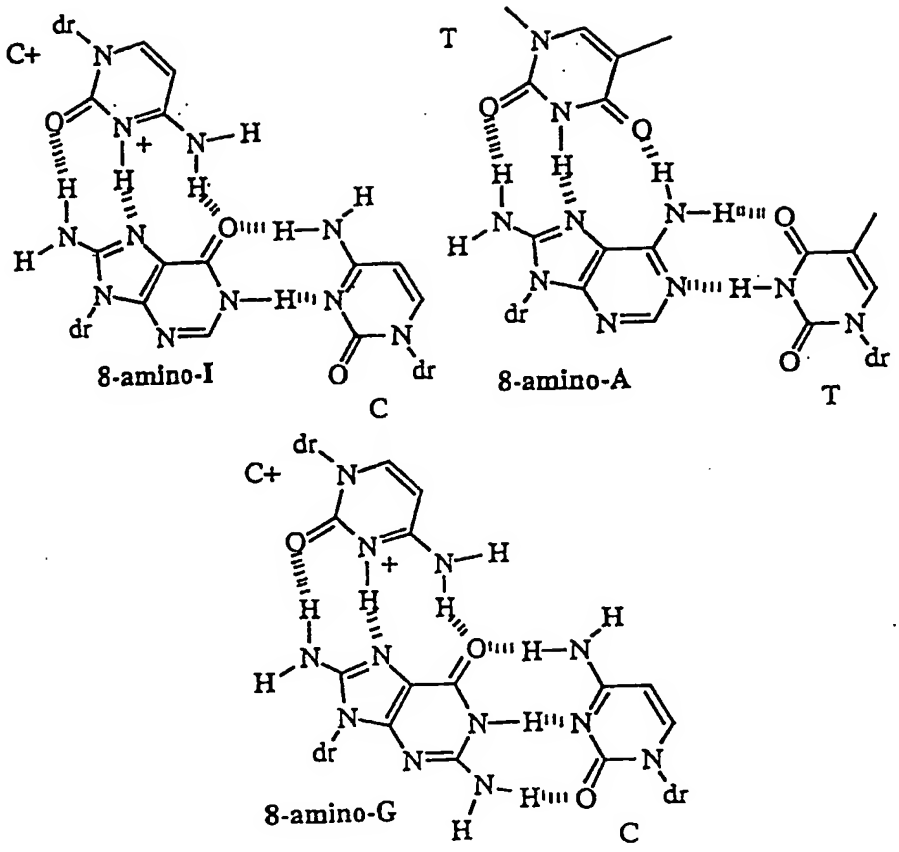



FIGURE 2

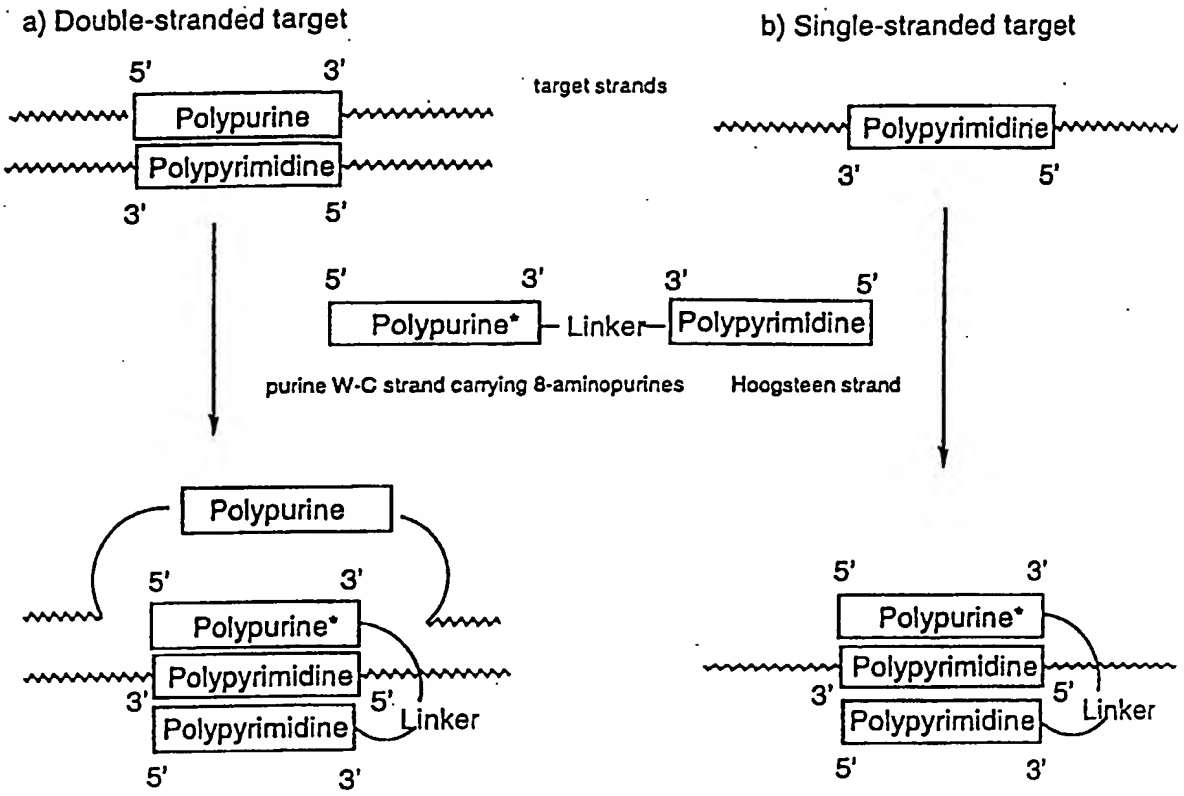

a) Double-stranded target
b) Single-stranded target
5'
3'
Polypurine
Polypyrimidine
target strands
Polypyrimidine
Polypurine*
Linker
purine W-C strand carrying 8-aminopurines
Hoogsteen strand

FIGURE 3

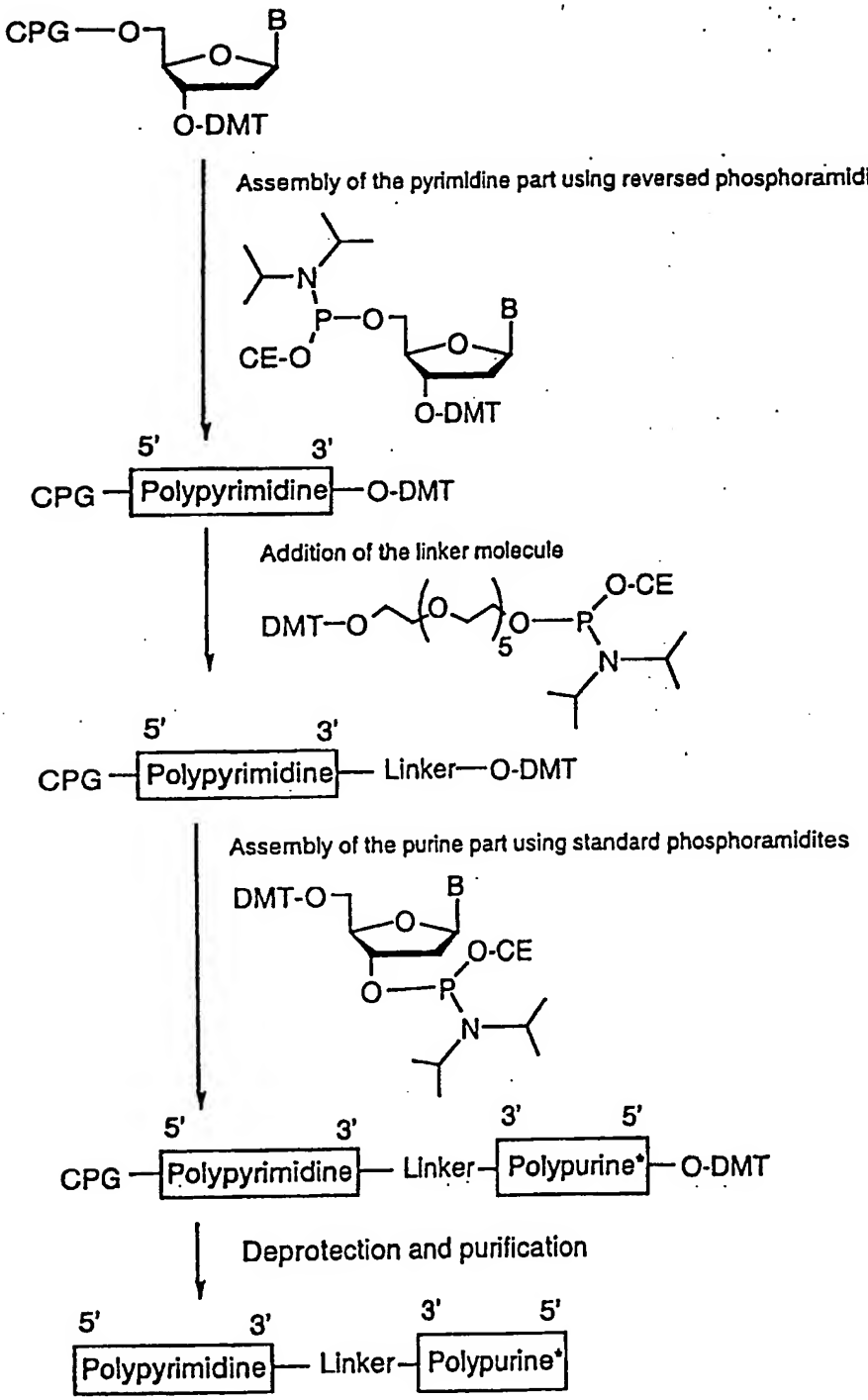
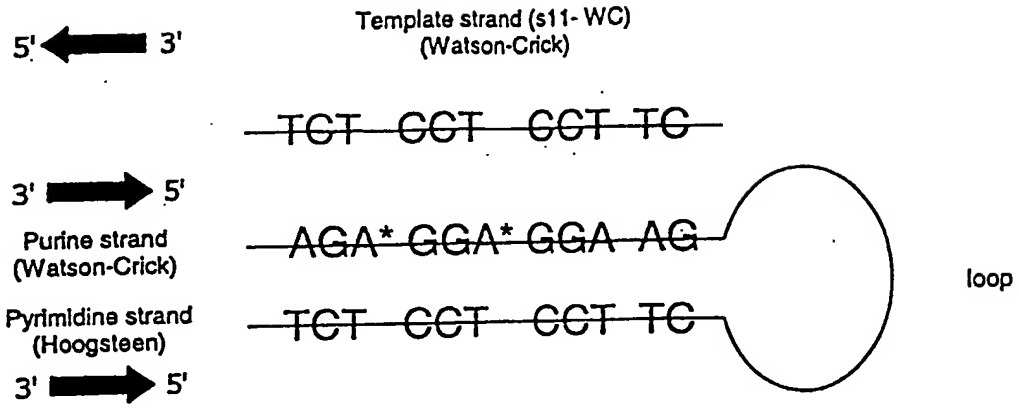
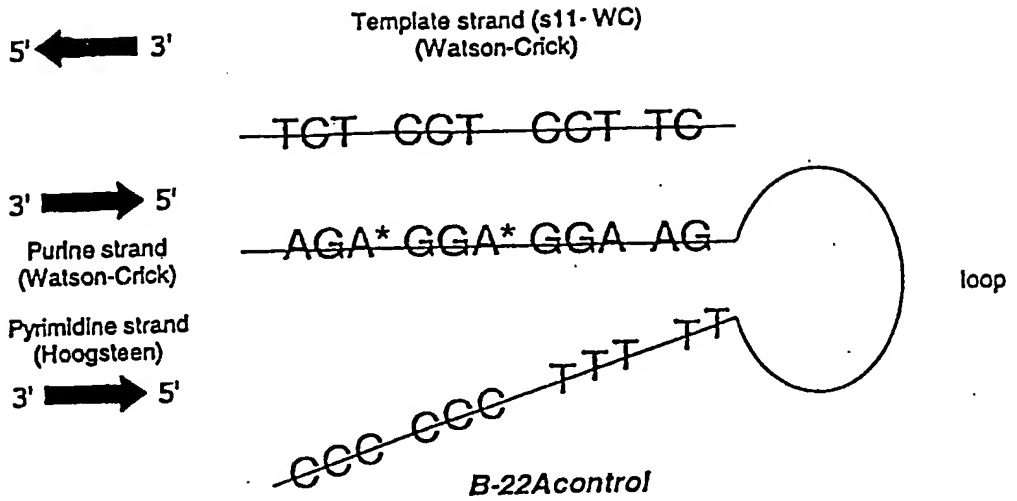

CPG
O
B
O
O-DMT
Assembly of the pyrimidine part using reversed phosphoramidites
N
P
O
CE-O
B
O
O-DMT
5'
3'
CPG
Polypyrimidine
O-DMT
Addition of the linker molecule
DMT-O
O
O
5
O
P
O-CE
N
5'
3'
CPG
Polypyrimidine
Linker
O-DMT
Assembly of the purine part using standard phosphoramidites
DMT-O
B
O
O-CE
O
P
N
5'
3'
3'
5'
CPG
Polypyrimidine
Linker
Polypurine*
O-DMT
Deprotection and purification
5'
3'
3'
5'
Polypyrimidine
Linker
Polypurine*

FIGURE 4

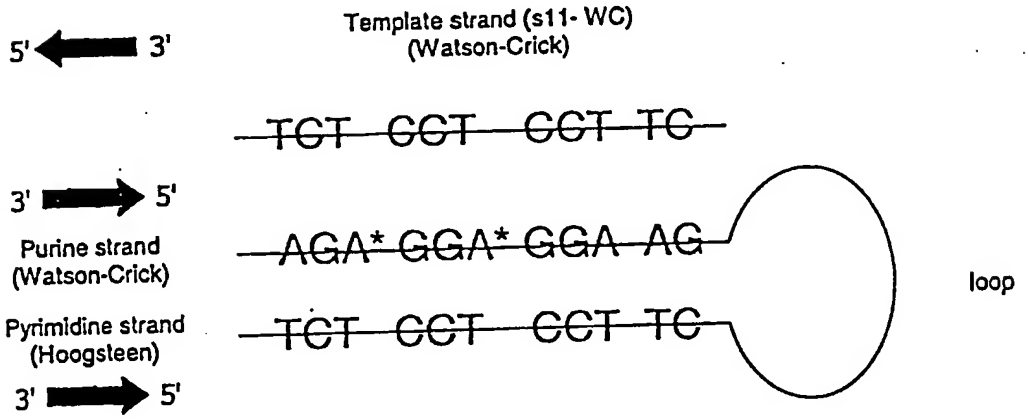



*B – 22 A*

**FIGURE 5**




*B-22Acontrol*




*B – 22 A*

FIGURE 6

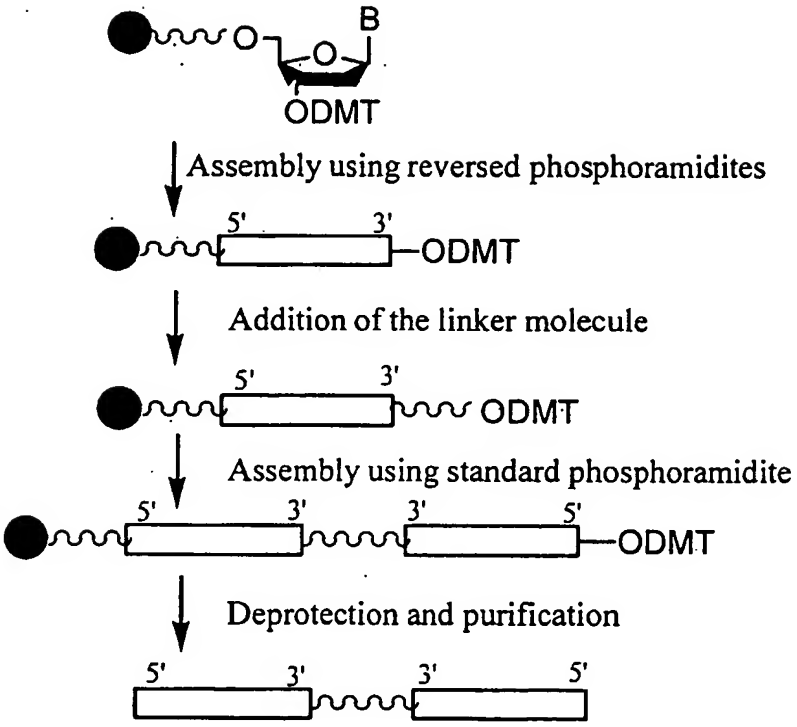

B
O
O
ODMT
Assembly using reversed phosphoramidites
5'
3'
ODMT
Addition of the linker molecule
5'
3'
ODMT
Assembly using standard phosphoramidite
5'
3'
3'
5'
ODMT
Deprotection and purification
5'
3'
3'
5'

Figure 7A

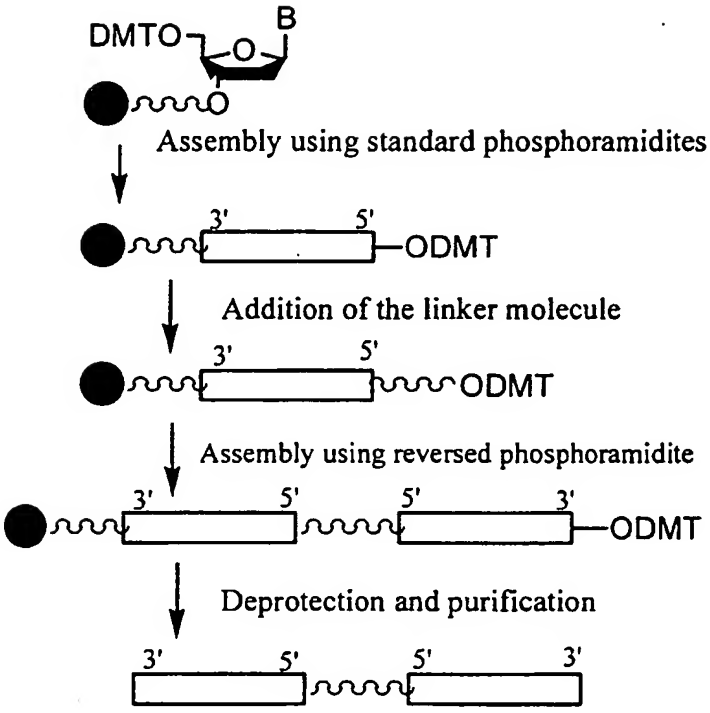

DMTO
B
O
O
Assembly using standard phosphoramidites
3'
5'
ODMT
Addition of the linker molecule
3'
5'
ODMT
Assembly using reversed phosphoramidite
3'
5'
5'
3'
ODMT
Deprotection and purification
3'
5'
5'
3'

Figure 7B

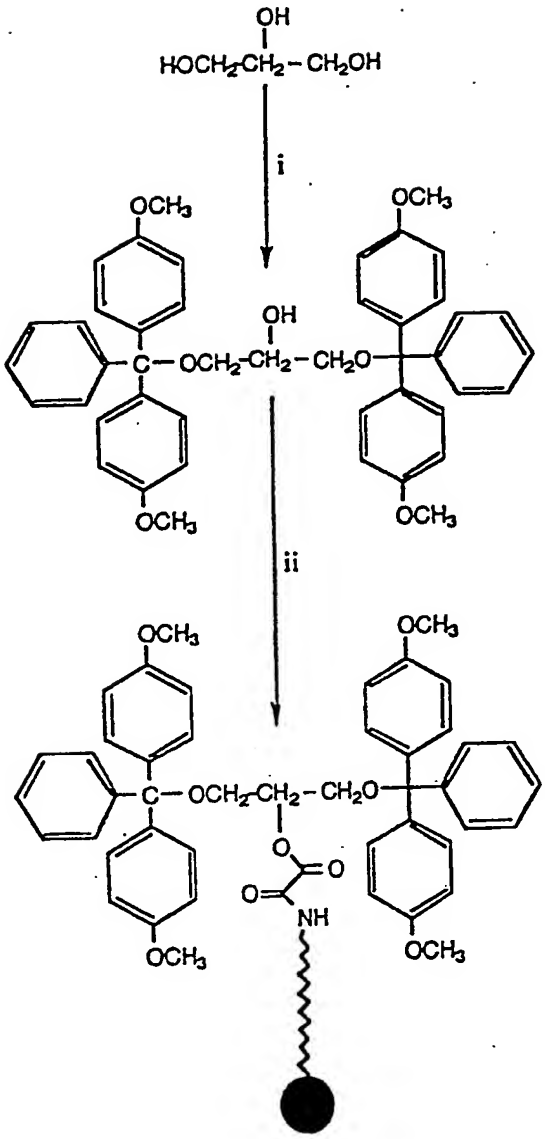

OH
HOCH2CH2-CH2OH
i
OCH3
OCH3
OH
C-OCH2-CH2-CH2O
OCH3
OCH3
ii
OCH3
OCH3
C-OCH2-CH2-CH2O
O
O
O
NH
OCH3
OCH3

Figure 8

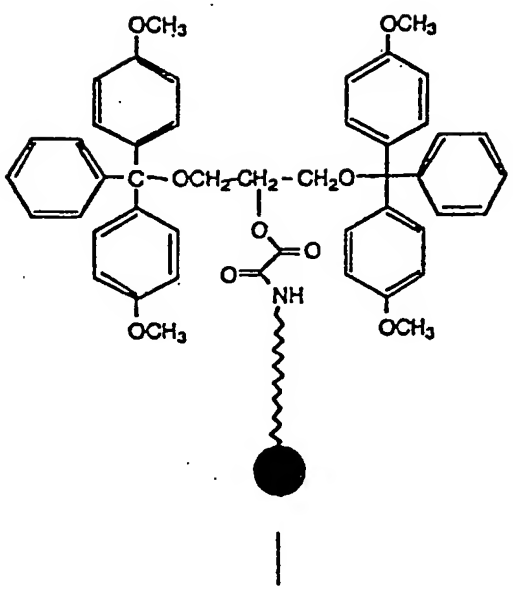



FIGURE 9A

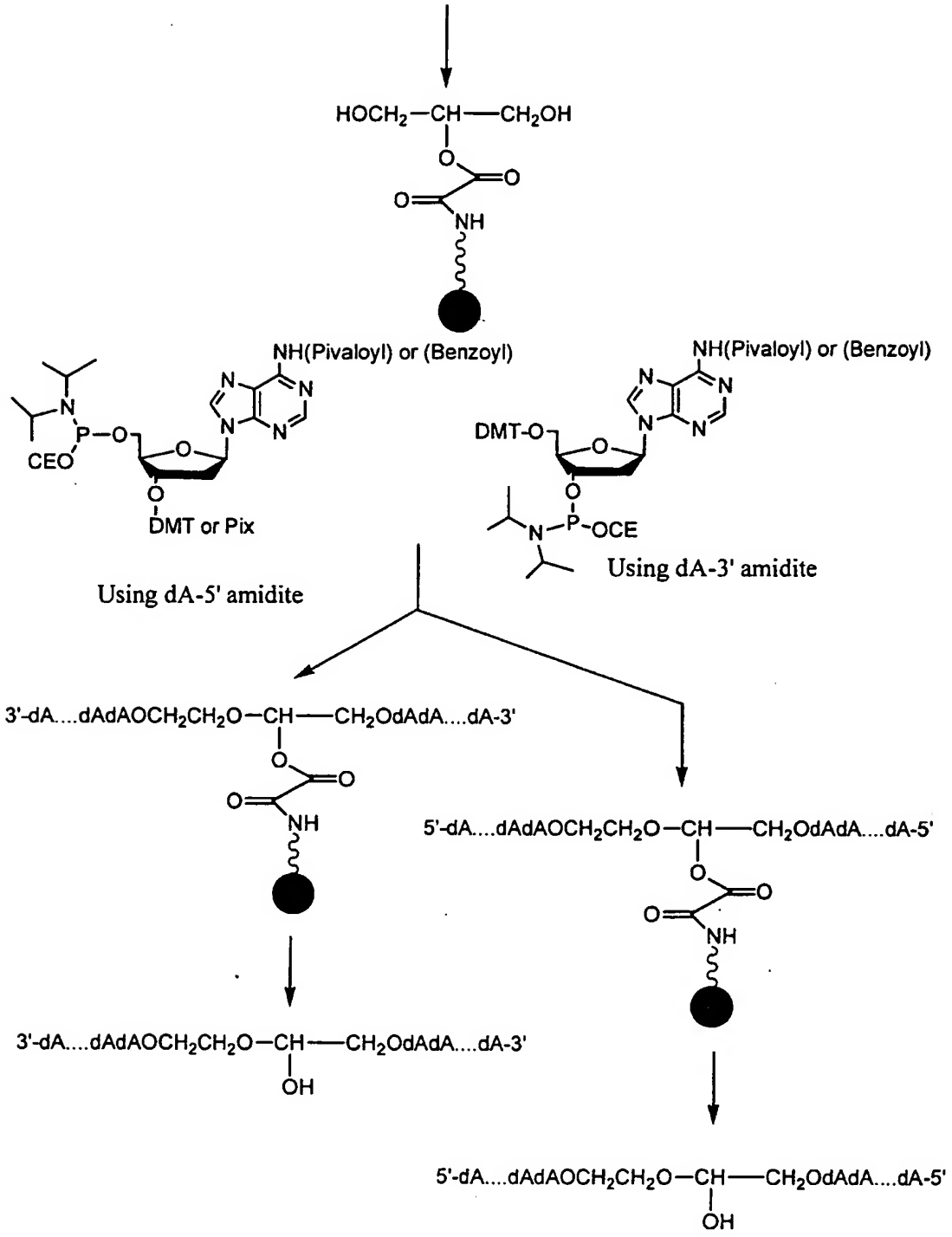

HOCH2—CH—CH2OH
O
O
O
NH
NH(Pivaloyl) or (Benzoyl)
CEO
DMT or Pix
Using dA-5' amidite
NH(Pivaloyl) or (Benzoyl)
DMT-O
OCE
Using dA-3' amidite
3'-dA....dAdAOCH2CH2O—CH—CH2OdAdA....dA-3'
5'-dA....dAdAOCH2CH2O—CH—CH2OdAdA....dA-5'
OH

Figure 9B

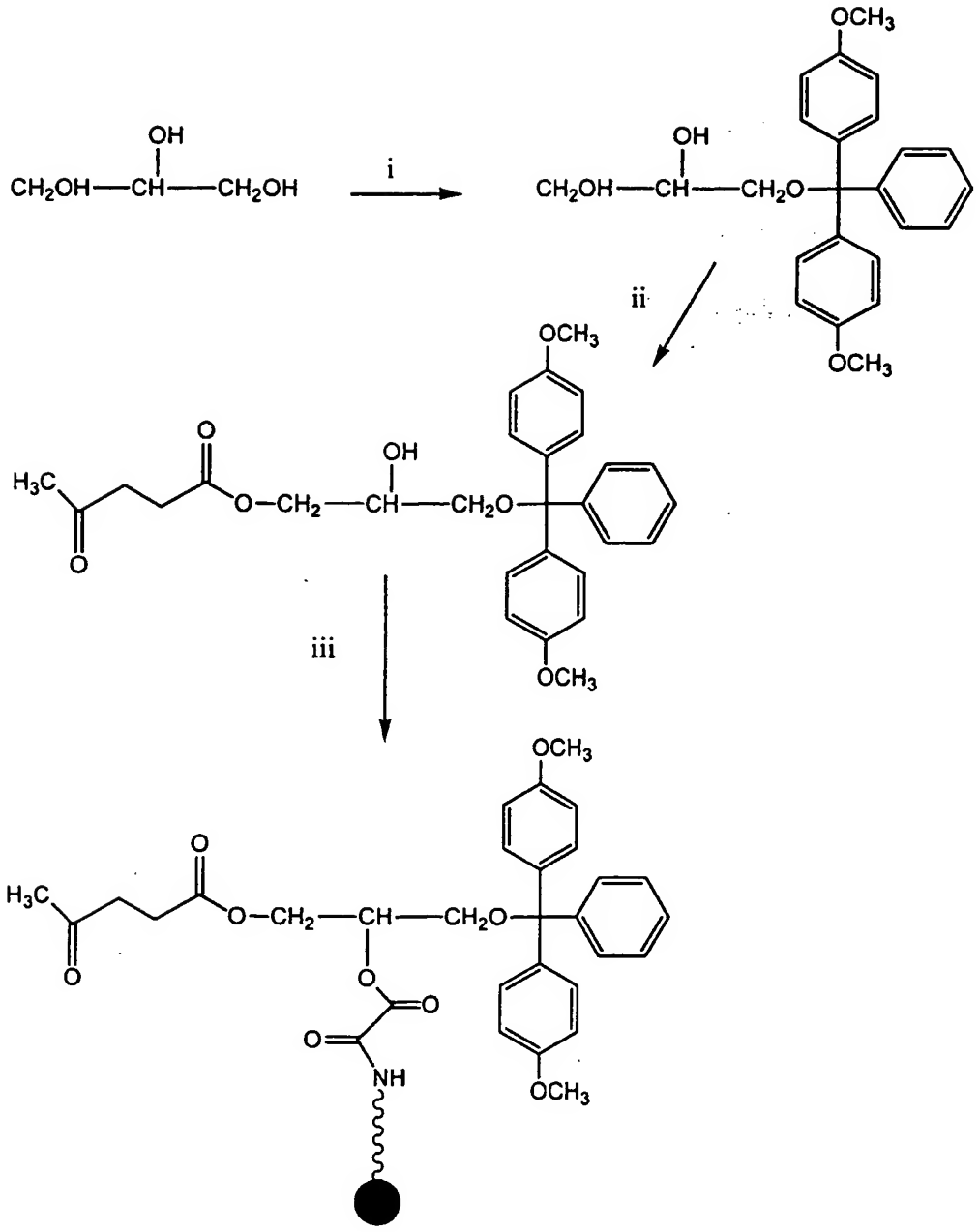



Figure 10

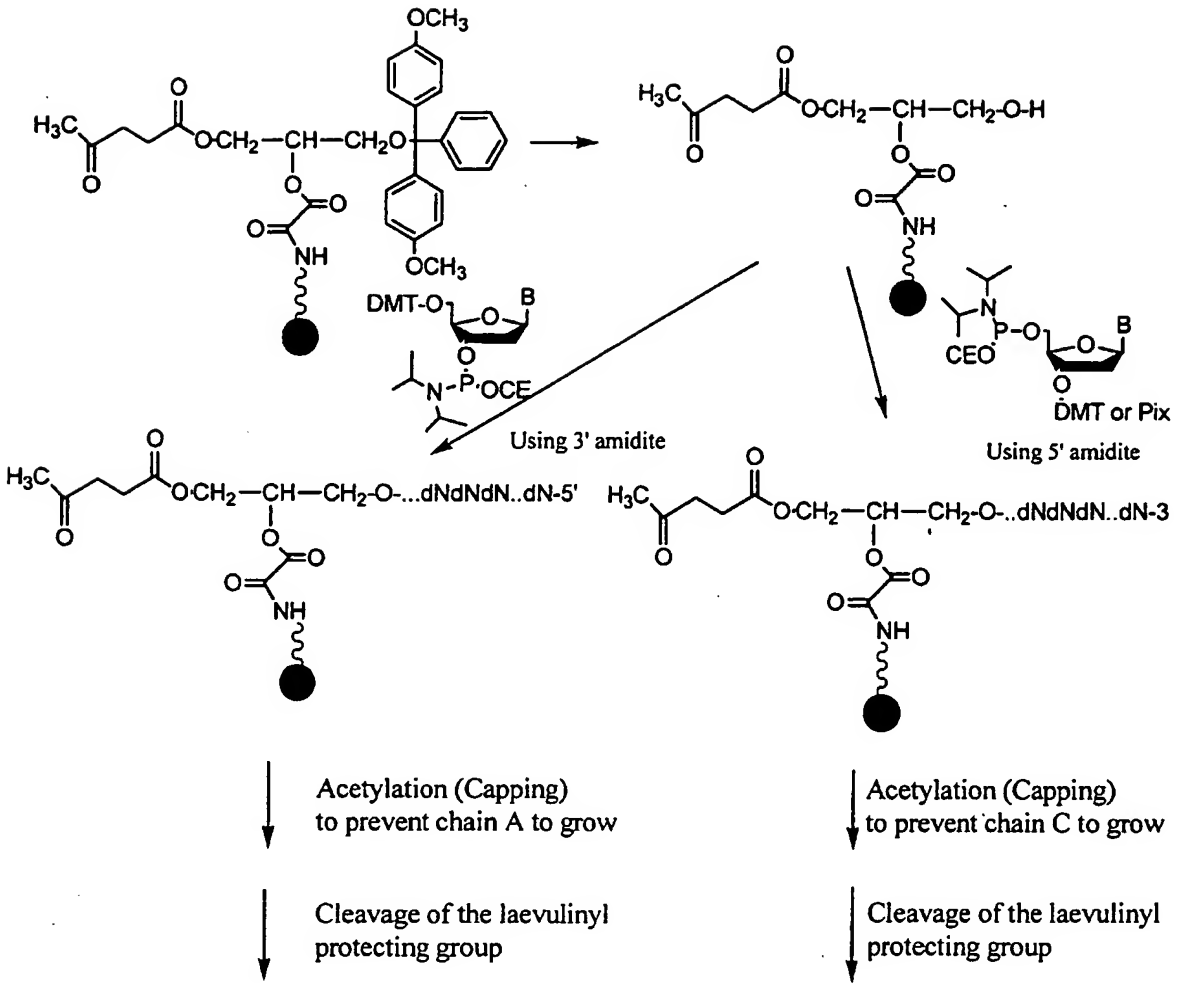

OCH3
H3C
O-CH2-CH-CH2-O
NH
DMT-O
B
N-P-OCE
CEO
DMT or Pix
Using 3' amidite
Using 5' amidite
O-CH2-CH-CH2-O-...dNdNdN..dN-5'
O-CH2-CH-CH2-O-..dNdNdN..dN-3
Acetylation (Capping)
to prevent chain A to grow
Acetylation (Capping)
to prevent chain C to grow
Cleavage of the laevulinyl
protecting group
Cleavage of the laevulinyl
protecting group

Figure 11A

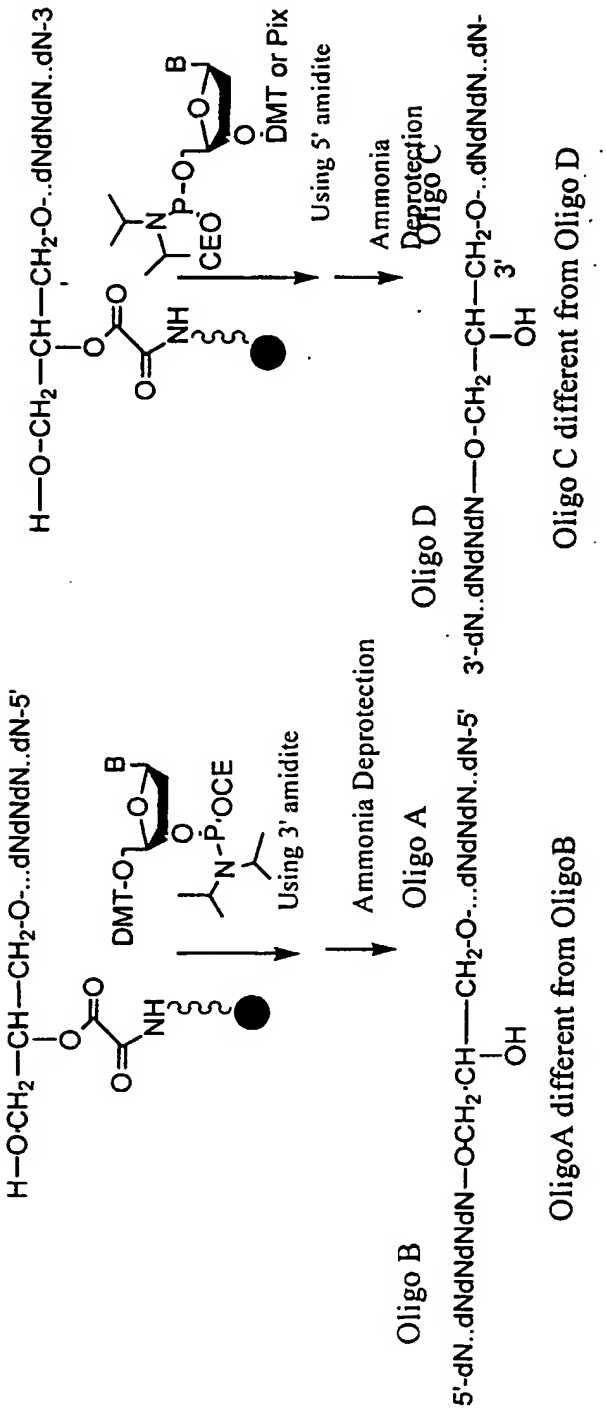

H—O-CH2—CH—CH2-O-...dNdNdN..dN-5'
DMT-O
B
O
O-P'OCE
N
Using 3' amidite
Ammonia Deprotection
Oligo A
Oligo B
5'-dN..dNdNdNdN—OCH2'CH—CH2-O-...dNdNdN..dN-5'
OH
OligoA different from OligoB
H—O-CH2—CH—CH2-O-..dNdNdN..dN-3
O
NH
CEO
P-O
B
DMT or Pix
Using 5' amidite
Ammonia Deprotection
Oligo C
Oligo D
3'-dN..dNdNdN—O-CH2—CH—CH2-O-..dNdNdN..dN-
3'
OH
Oligo C different from Oligo D

Figure 11B

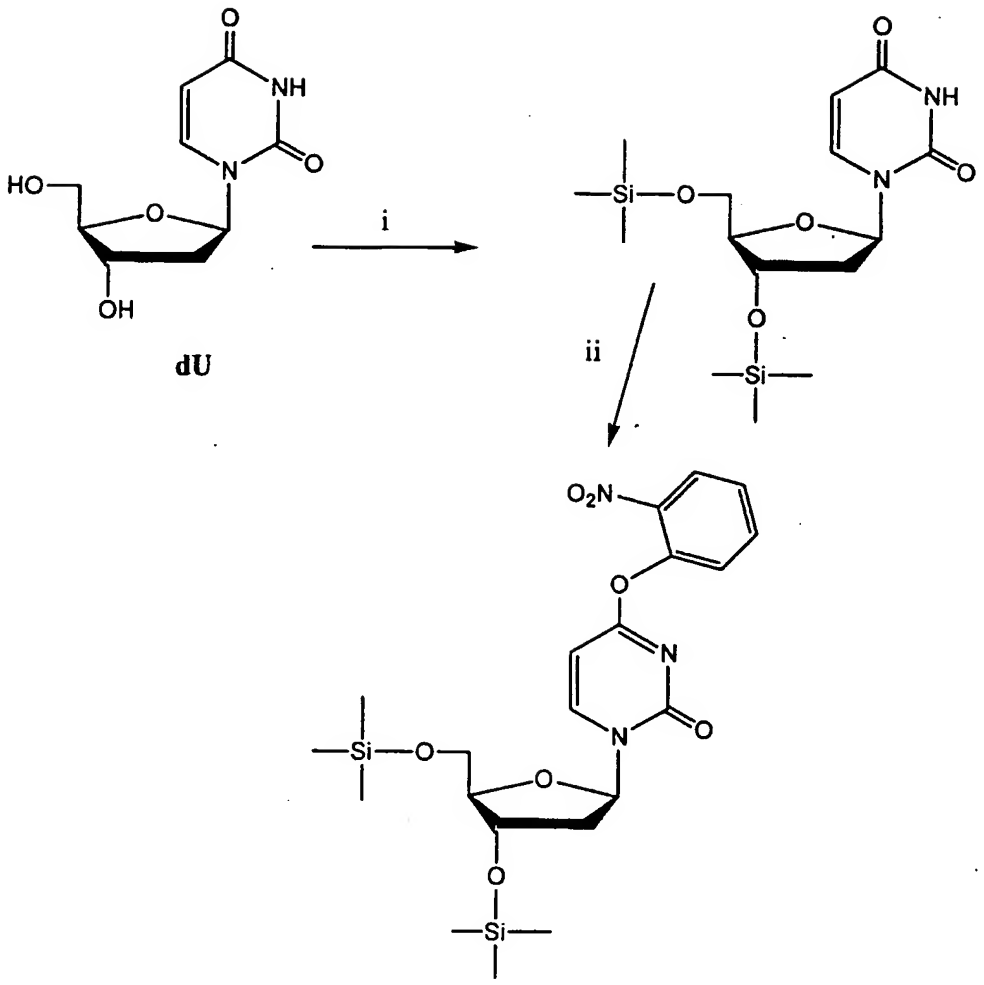



**FIGURE 12**

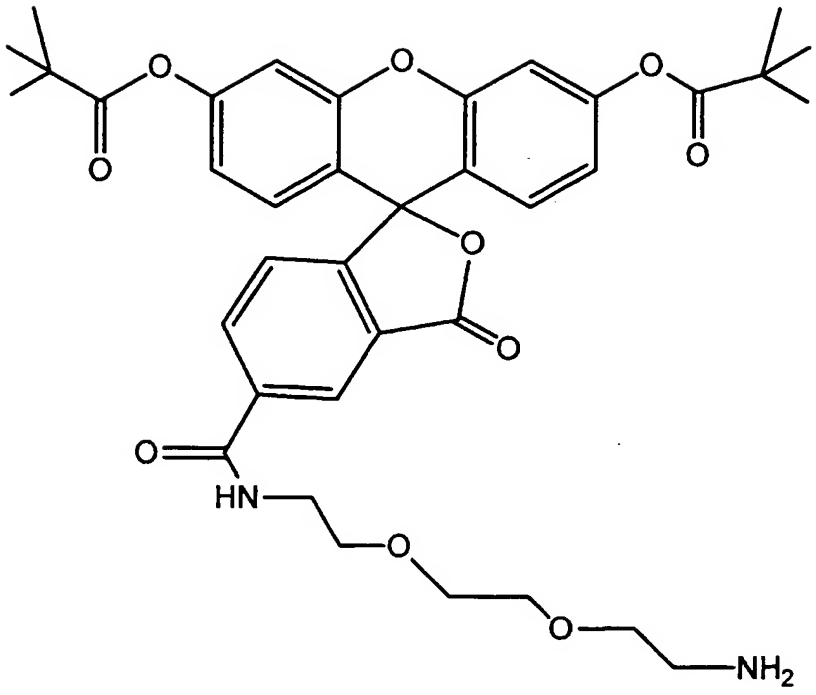



Figure 13

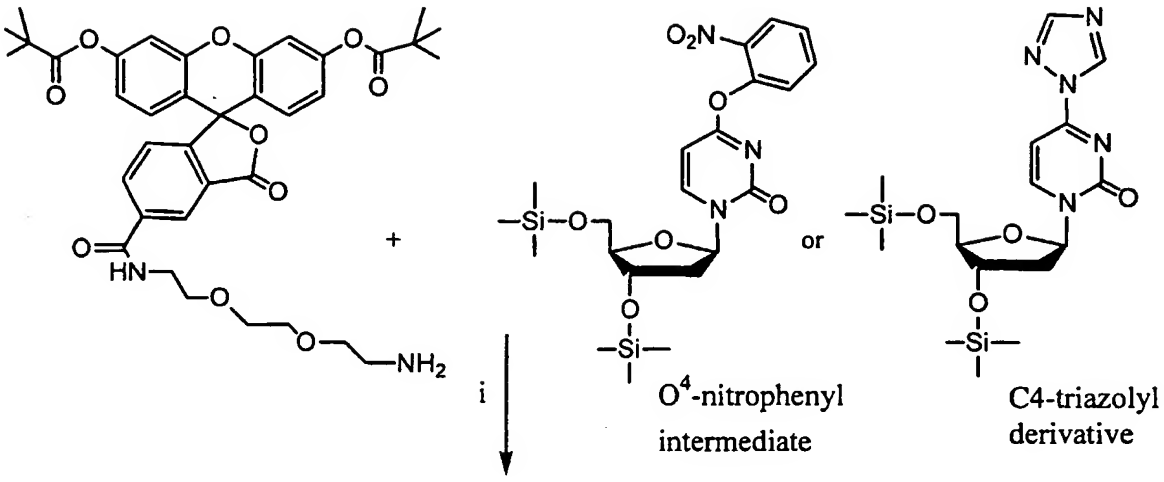

HN
NH2
i
or
O4-nitrophenyl
intermediate
C4-triazolyl
derivative

Figure 14A

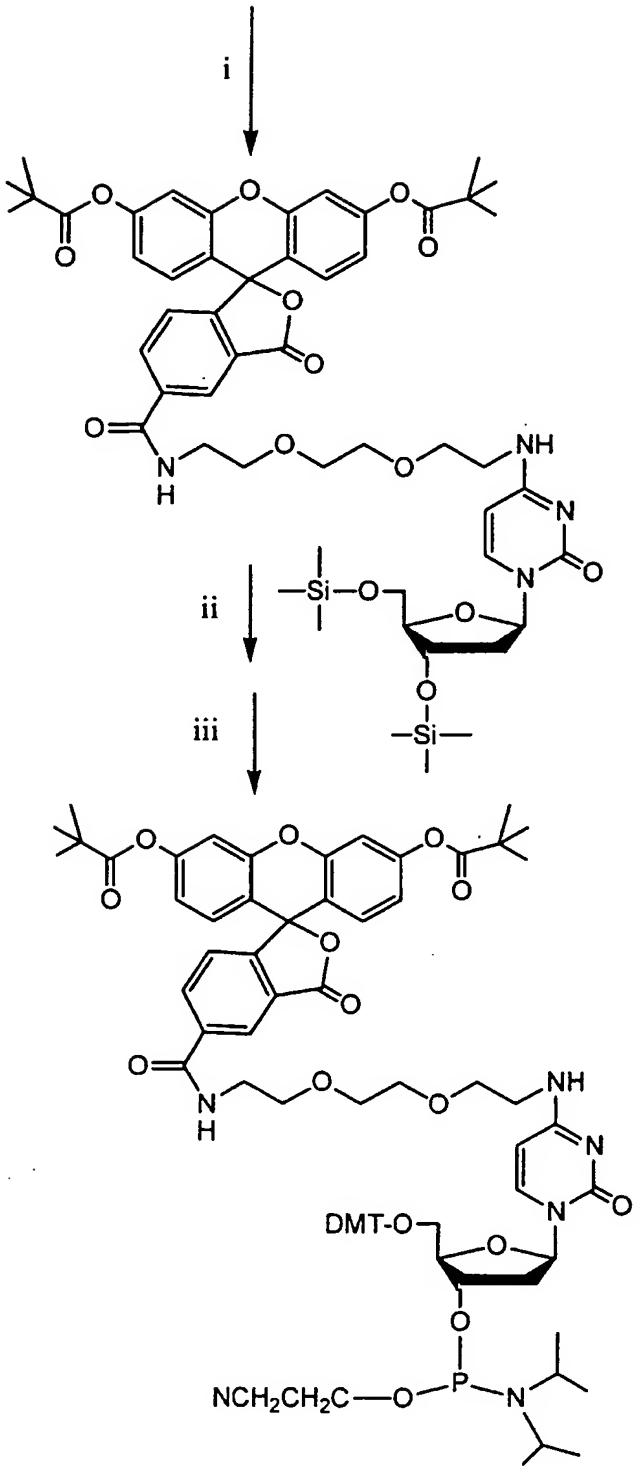



Figure 14B

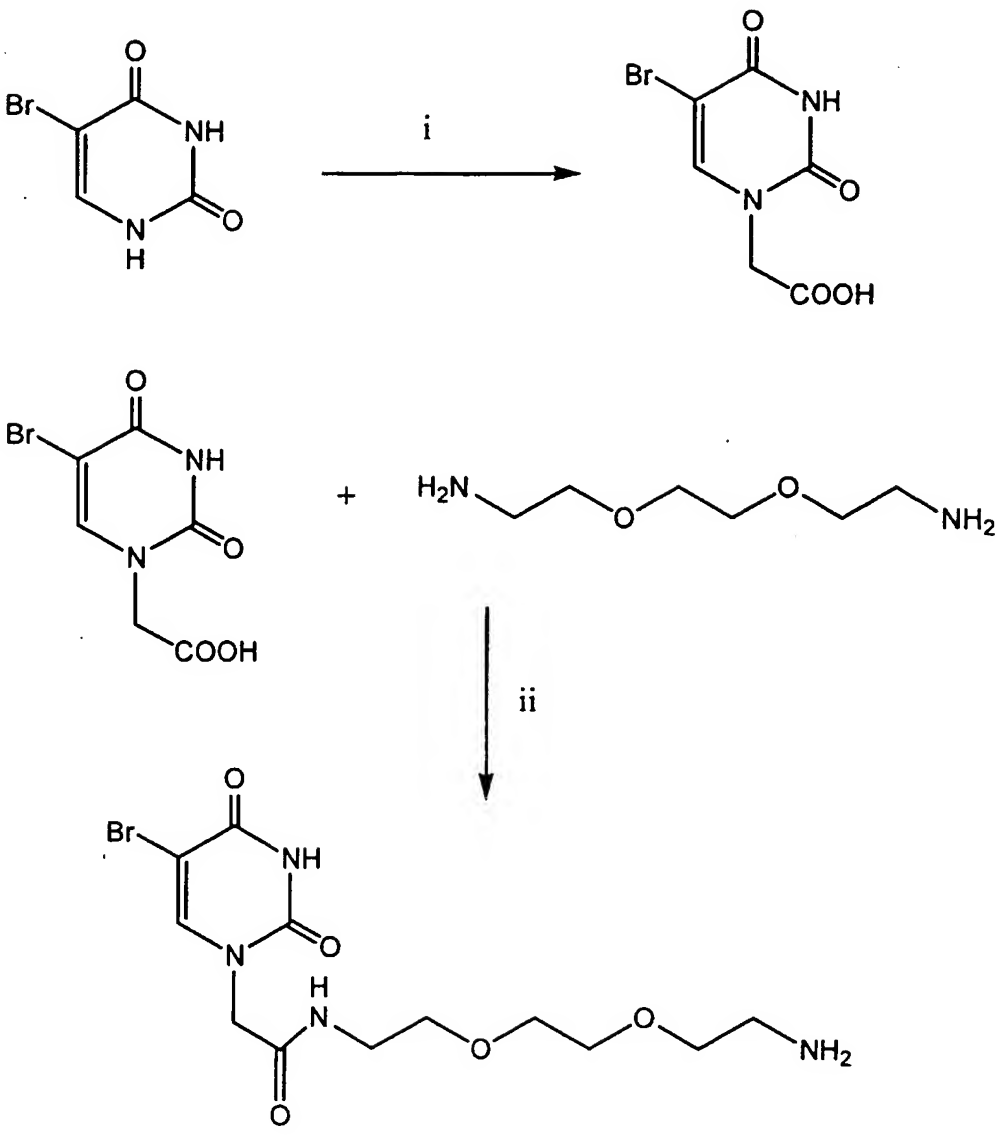

Br
O
NH
N
H
O
i
Br
O
NH
N
O
COOH
Br
O
NH
N
O
COOH
+
H2N
O
O
NH2
ii
Br
O
NH
N
O
H
N
O
O
NH2
O

Figure 15

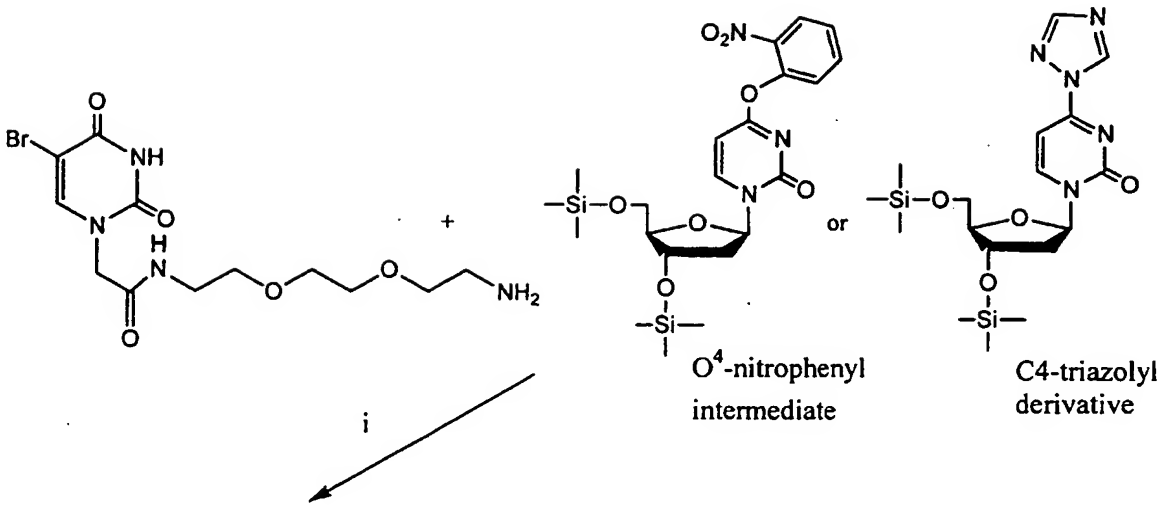

Br
NH
O
N
H
N
O
O
O
O
NH2
+
i
O2N
O
N
O
N
O
Si
O
O
Si
O4-nitrophenyl
intermediate
or
N
N
N
N
O
Si
O
O
Si
C4-triazolyl
derivative

Figure 16A

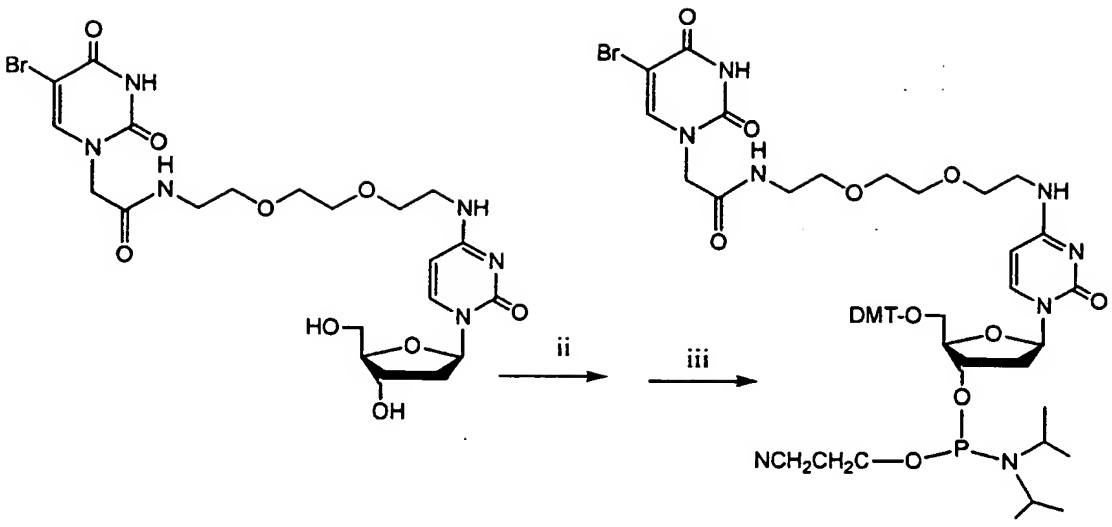



Figure 16B

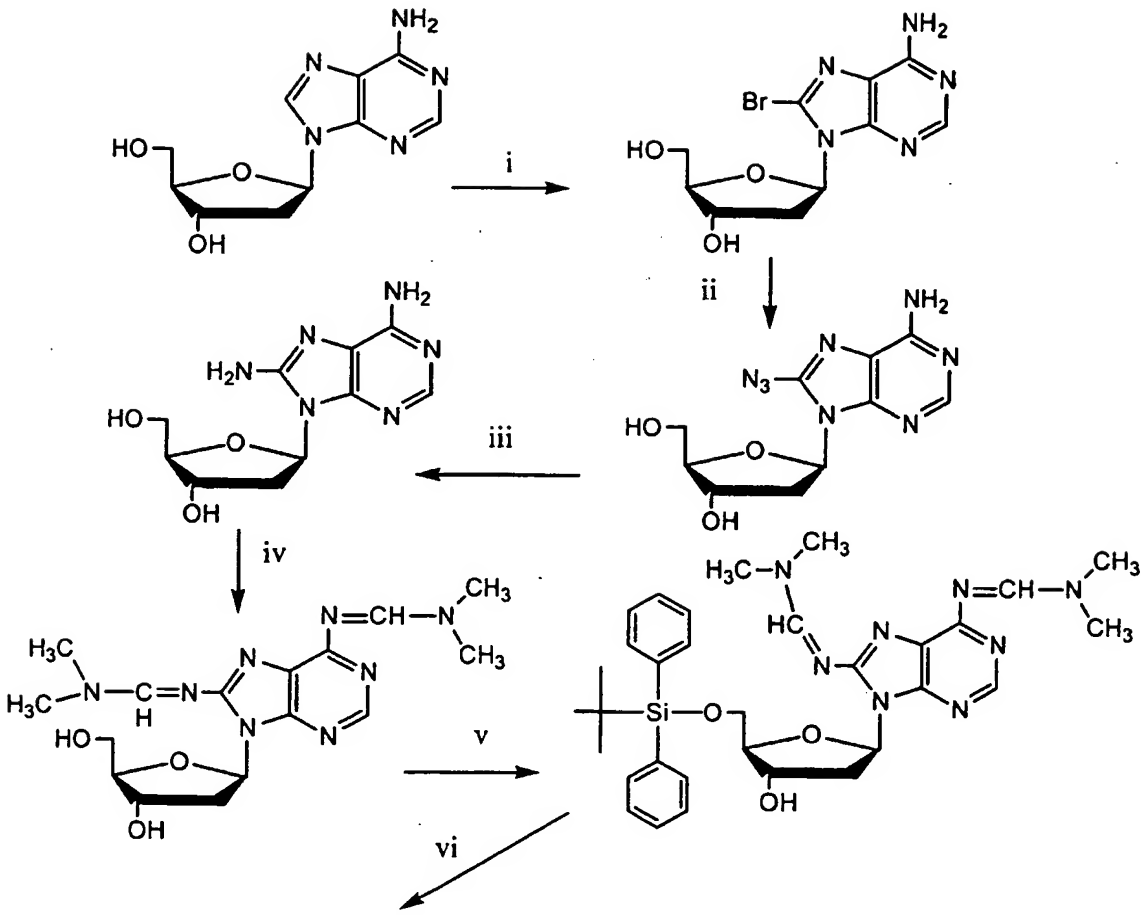



Figure 17A

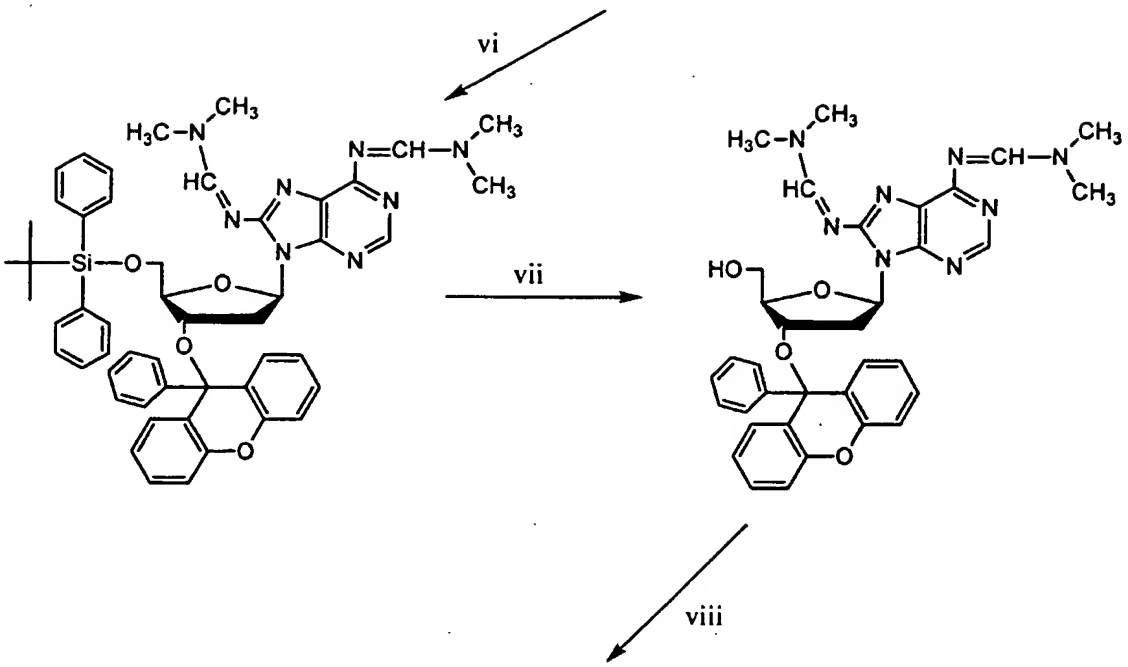



Figure 17B

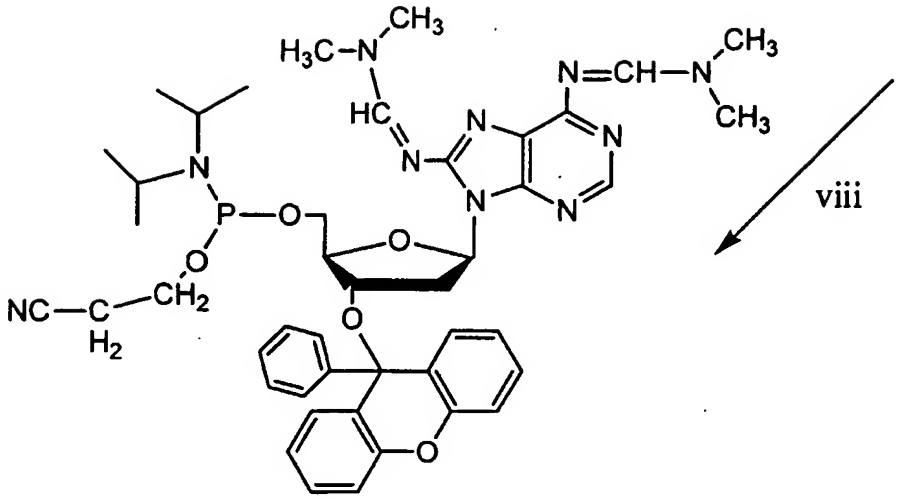



Figure 17C

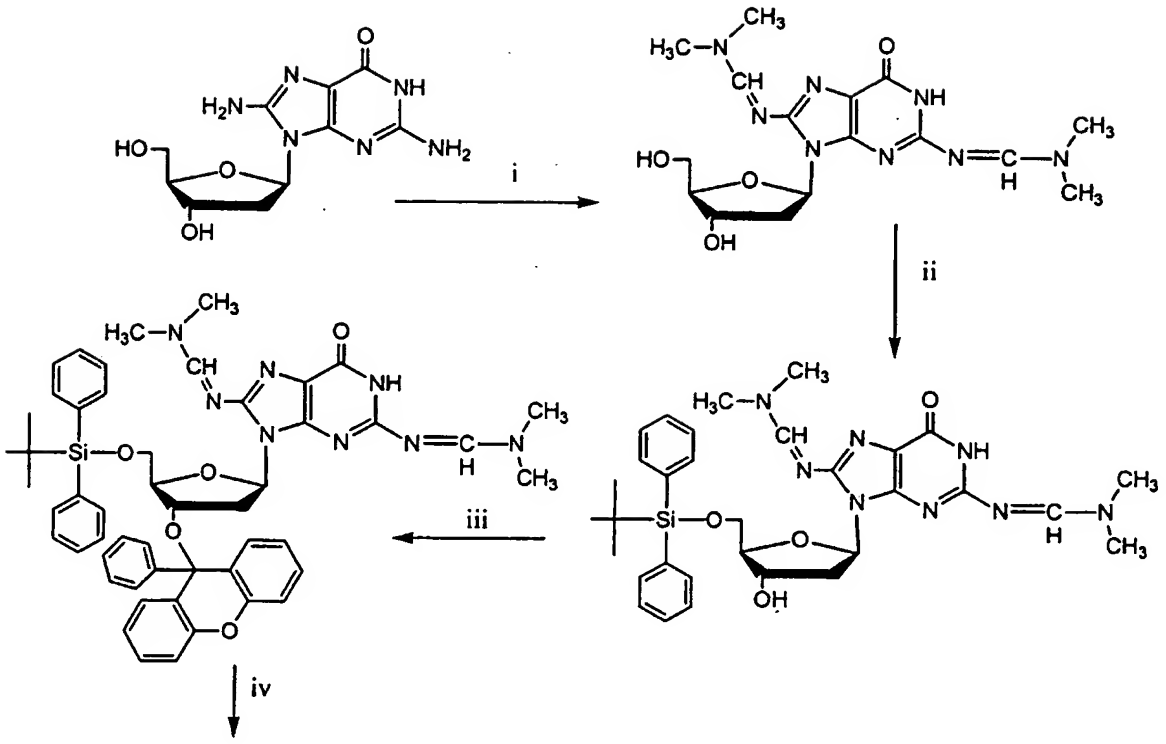



Figure 18A

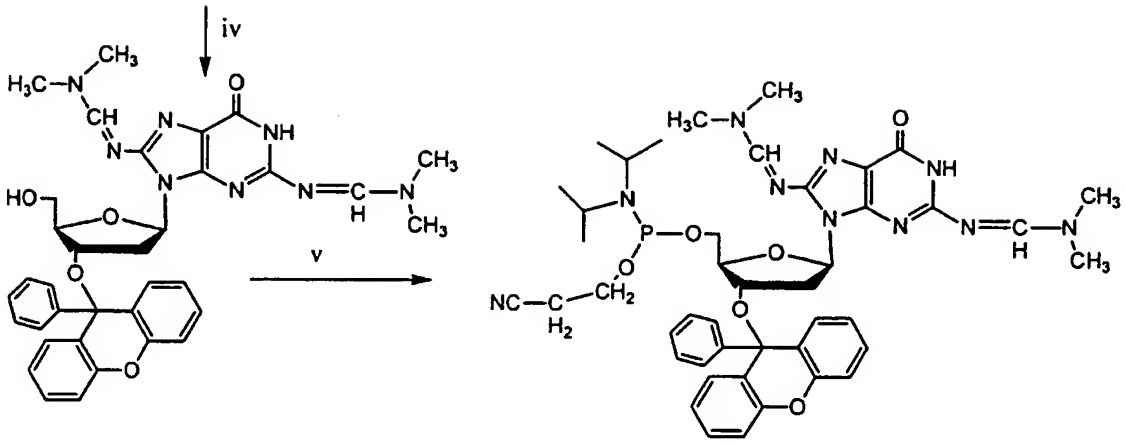



Figure 18B

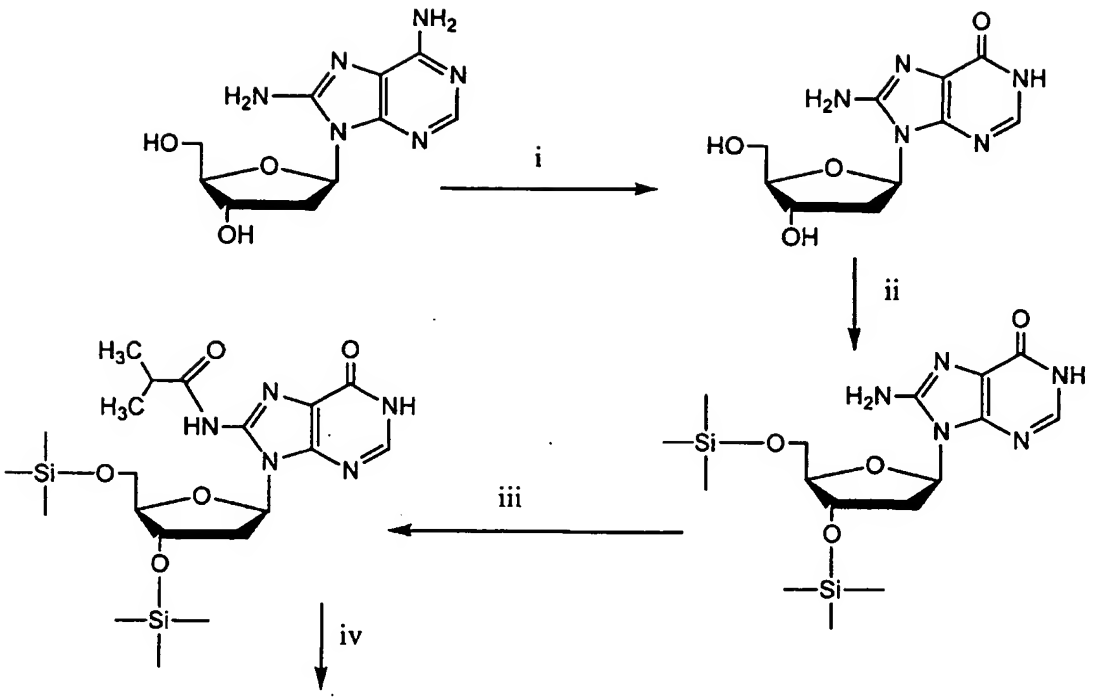



Figure 19A

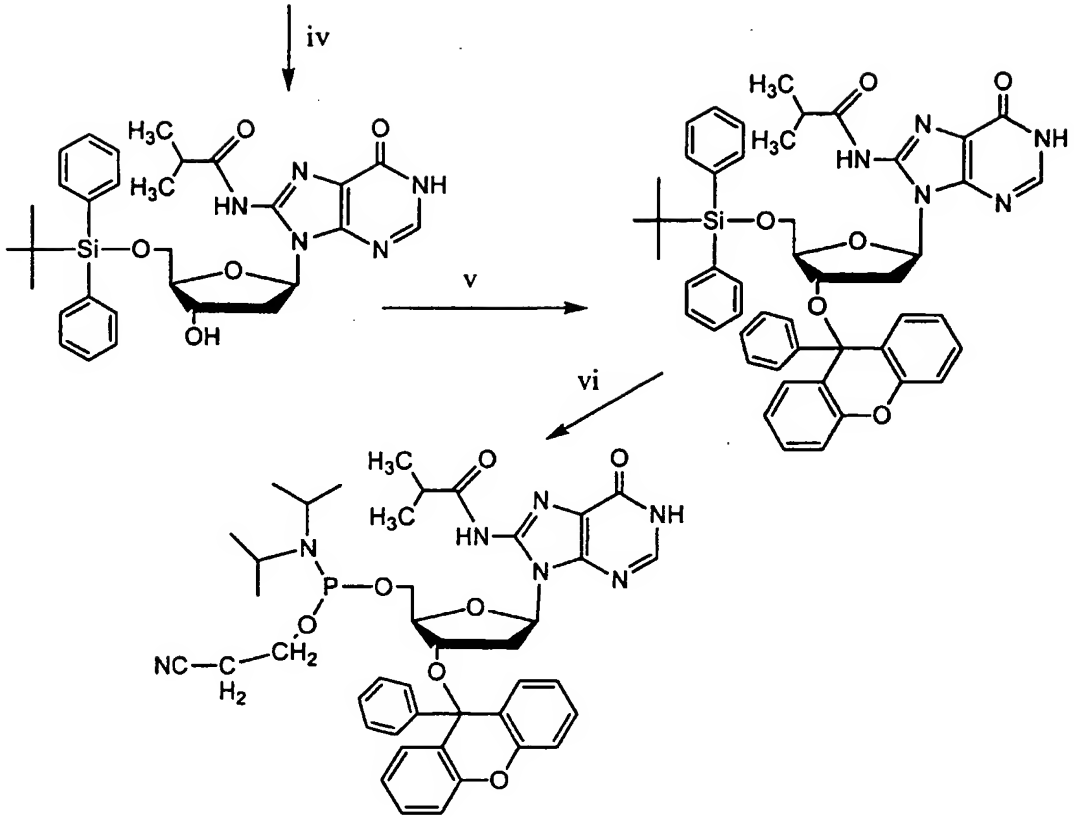



Figure 19B

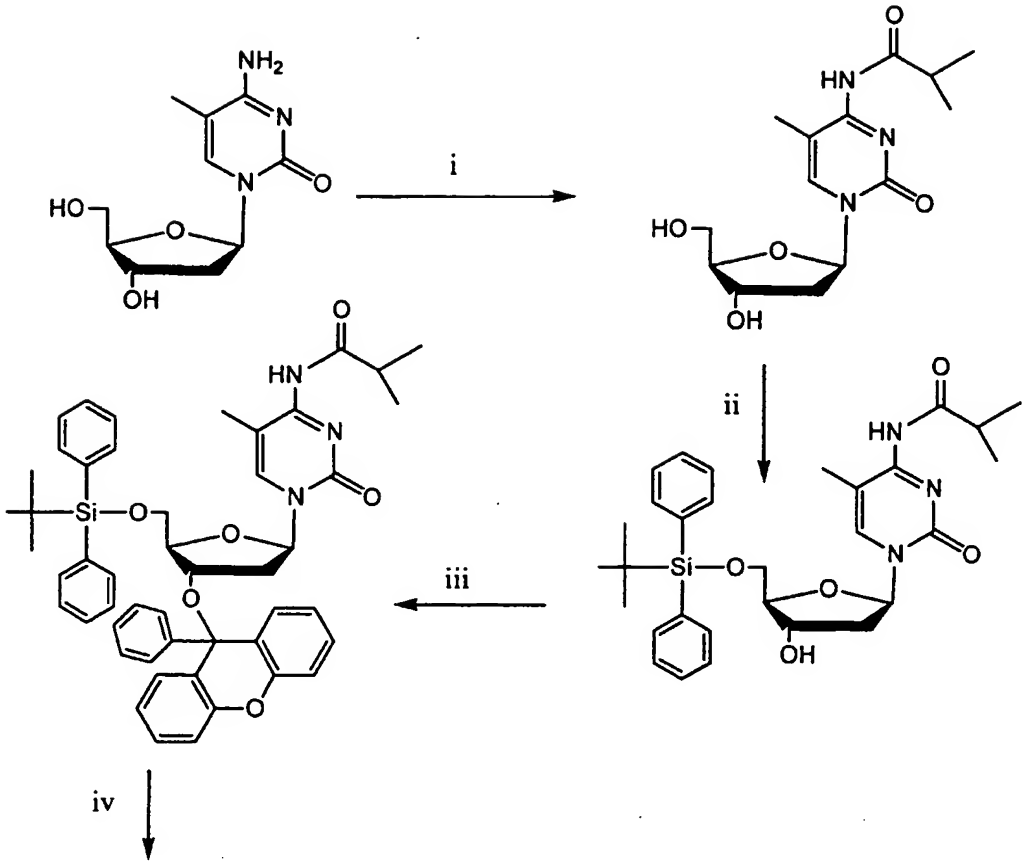

NH2
N
N
O
HO
O
OH
i
O
HN
N
N
O
HO
O
OH
ii
O
HN
N
N
O
Si
O
O
iii
O
HN
N
N
O
Si
O
O
O
O
iv

Figure 20A

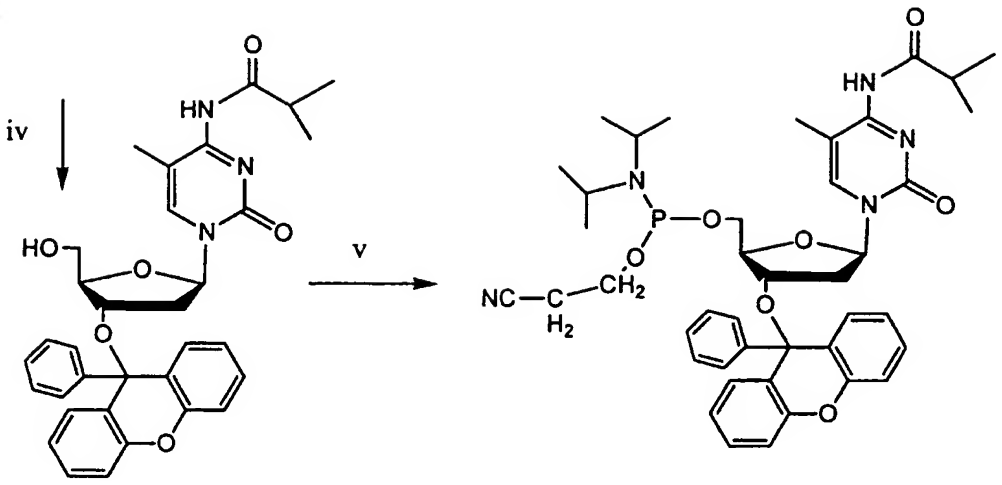



Figure 20B

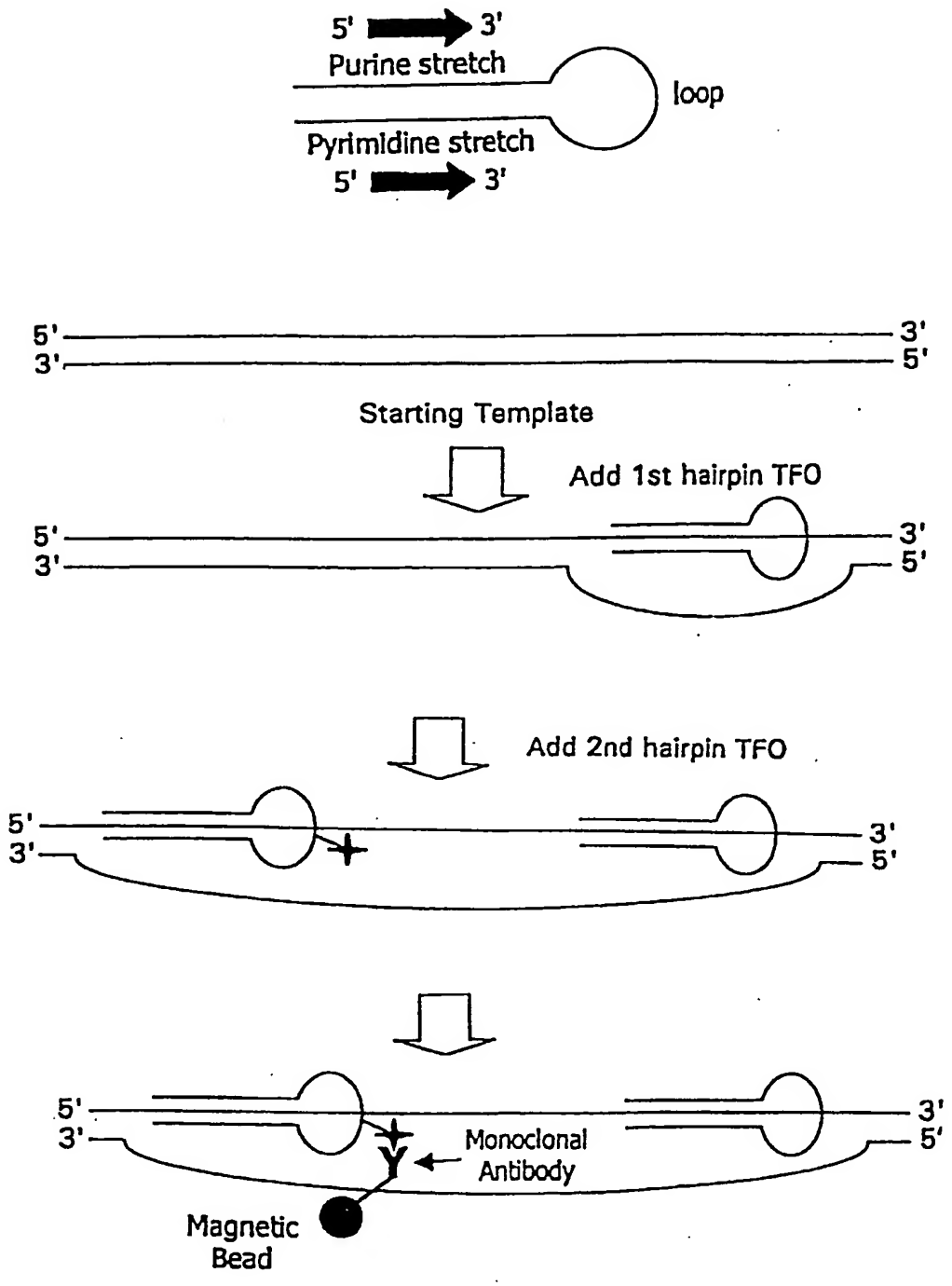
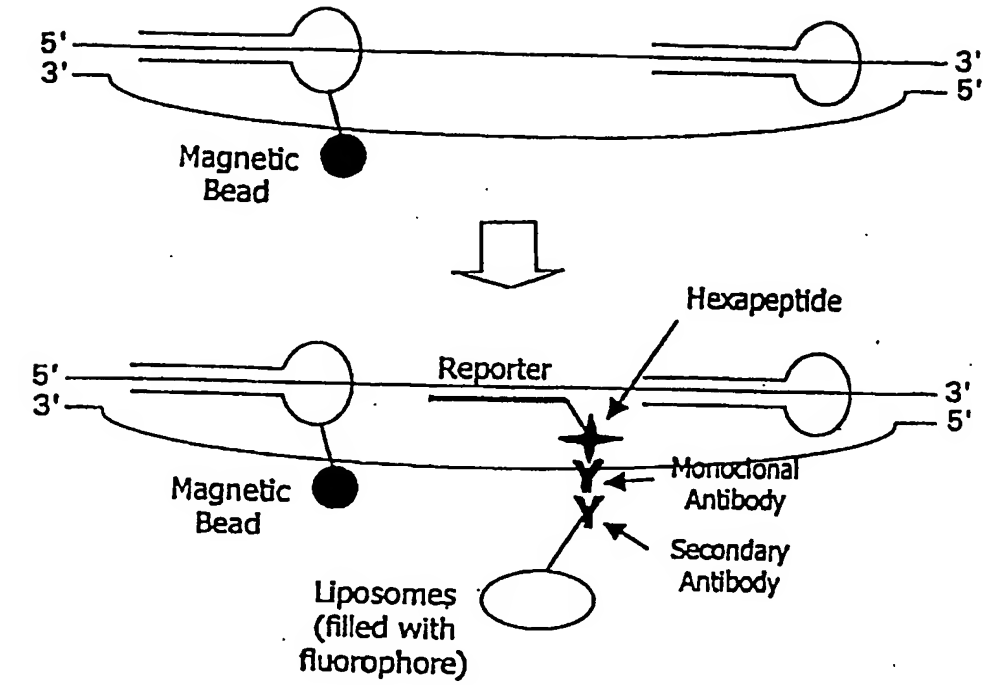

5'
3'
Purine stretch
loop
Pyrimidine stretch
5'
3'
5'
3'
3'
5'
Starting Template
Add 1st hairpin TFO
5'
3'
3'
5'
Add 2nd hairpin TFO
5'
3'
3'
5'
5'
3'
Monoclonal
Antibody
Magnetic
Bead
3'
5'

FIGURE 21A


5'
3'
3'
5'
Magnetic Bead
Hexapeptide
Reporter
Monoclonal Antibody
Secondary Antibody
Liposomes (filled with fluorophore)

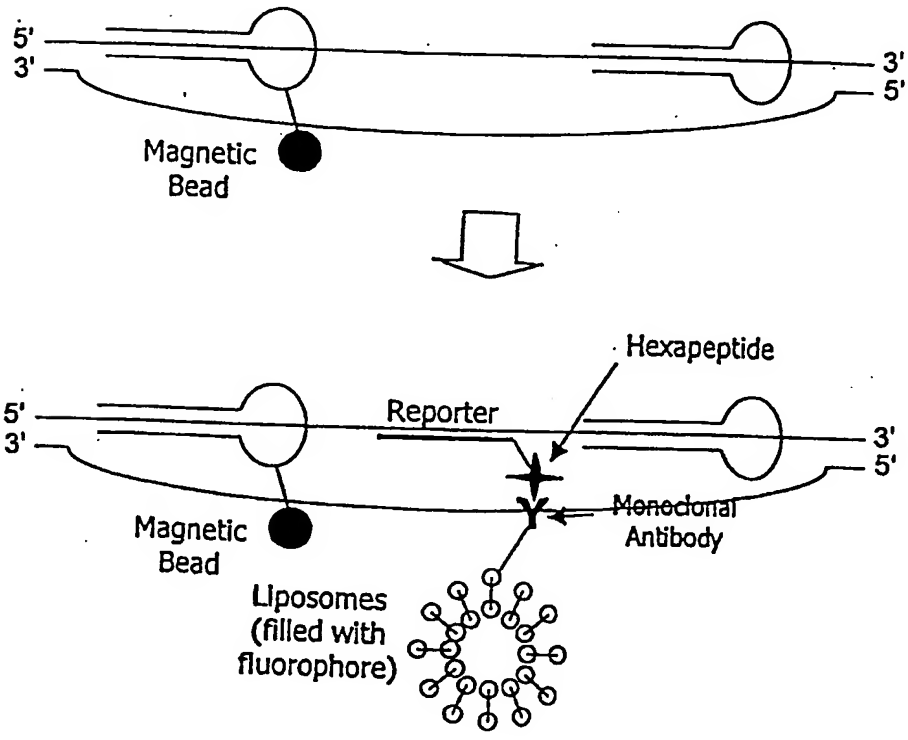

5'
3'
3'
5'
Magnetic Bead
Hexapeptide
Reporter
Monoclonal Antibody
Liposomes (filled with fluorophore)

FIGURE 21B

# INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US00/29918

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; C07H 19/00, 21/00, 21/02, 21/04
US CL :435/6; 536/22.1, 23.1 24.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/22.1, 23.1 24.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN
search terms: triplex DNA, polypyrimidine, polypurine, stability

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 5,422,251 A (FRESCO et al.) 6 June 1995, see entire document. | 1-20 |
| Y | US 5,739,308 A (KANDIMALLA et al.) 14 Aprile 1998, see entire document. | 1-20 |
| Y | LEE et al. Poly(pyrimidien).poly(purine) Synthetic DNAs Containing 5-Methylcytosine Form Stable Triplexes at Neutral pH. Nucleic Acids Research. 1984. Vol. 12. No. 16. pages 6603-6613, see entire document. | 1-20 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
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